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THE UNIVERSITY OF ALBERTA

PROPERTIES AND KINETIC STUDIES OF OROTIDYLATE DECARBOXYLASE

by

© LAURITZ P. SIMONSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL, 1973

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Properties and Kinetic Studies of Orotidylate Decarboxylase" submitted by Lauritz P. Simonson in partial fulfilment of the requirements for the degree of Master of Science.

.....
Supervisor

.....

.....

.....
External Examiner

October 1, 1973
Date

ABSTRACT

Orotidylate decarboxylase was purified to near homogeneity by a five-step procedure. Sensitivity to sulfhydryl reagents and the absence of a Mg^{++} requirement for activity were confirmed. The sensitivity to sulfhydryl reagents appears to involve a protein conformational change rather than the blocking of an active sulfhydryl group of the catalytic site. Two sulfhydryl groups of the protein appear to be freely accessible to the solvent.

A molecular weight of about 150,000 daltons was determined for the protein, which in the presence of sodium dodecyl sulfate breaks down to eight subunits of apparently identical molecular weight. Active subunits show no cooperativity or interaction. The value of the Michaelis constant of orotidylate is only slightly greater than its dissociation constant under all conditions tested.

Reversibility of the reaction was demonstrated with the aid of a product trap. Product inhibition studies with uridylate and bicarbonate appeared to favor a reaction mechanism where uridylate is released before bicarbonate with uridylate being capable of binding to the central complex to form a dead end complex.

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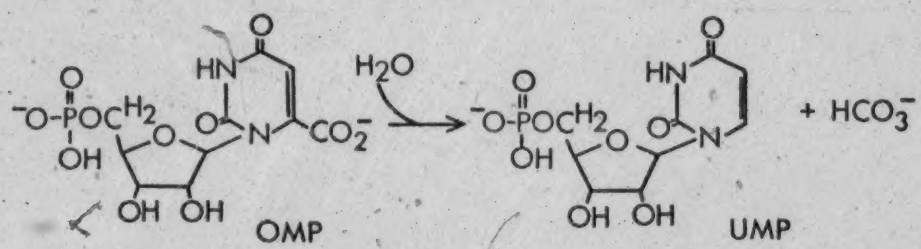
LIST OF ABBREVIATIONS

UMP	uridine 5'-monophosphate
OMP	orotidine 5'-monophosphate
NEM	N-ethylmaleimide
IAA	iodoacetamide
pCMB	para-chloromercuribenzoate
AMP	adenosine 5'-monophosphate
CMP	cytidine 5'-monophosphate
GMP	guanosine 5'-monophosphate
CDP	cytidine 5'-diphosphate
UDP	uridine 5'-diphosphate
CTP	cytidine 5'-triphosphate
UTP	uridine 5'-triphosphate
IMP	inosine 5'-monophosphate
6-aza-UMP	6 azauridine 5'-monophosphate
KP	potassium phosphate
HCO_3^-	bicarbonate
EDTA	ethylenediaminetetracetate
NH_4COOH	ammonium formate
Km	Michaelis constant
K_d	dissociation constant
nm	nanometers
A_{280}	optical density at 280 nanometers
ml	milliliter
ul	microliter
V_e	elution volume
V_o	void volume
V	velocity
rpm	revolutions per minute
M	moles per liter
ma	milliamperes
ug	microgram
g	gravity
u	micron
tris	tris(hydroxylmethyl)aminomethane
P_i	inorganic phosphate
PRPP	5-phosphoribosyl 1-pyrophosphate
NAD	nicotinamide adenine dinucleotide
PP	pyrophosphate
ATP	adenosine 5'-triphosphate
ADP	adenosine 5'-diphosphate
cm	centimeter

I. INTRODUCTION

The enzyme orotidylate decarboxylase (orotidine 5' phosphate carboxy-lyase, E.C. 4.1.1.23) catalyzes the reaction:

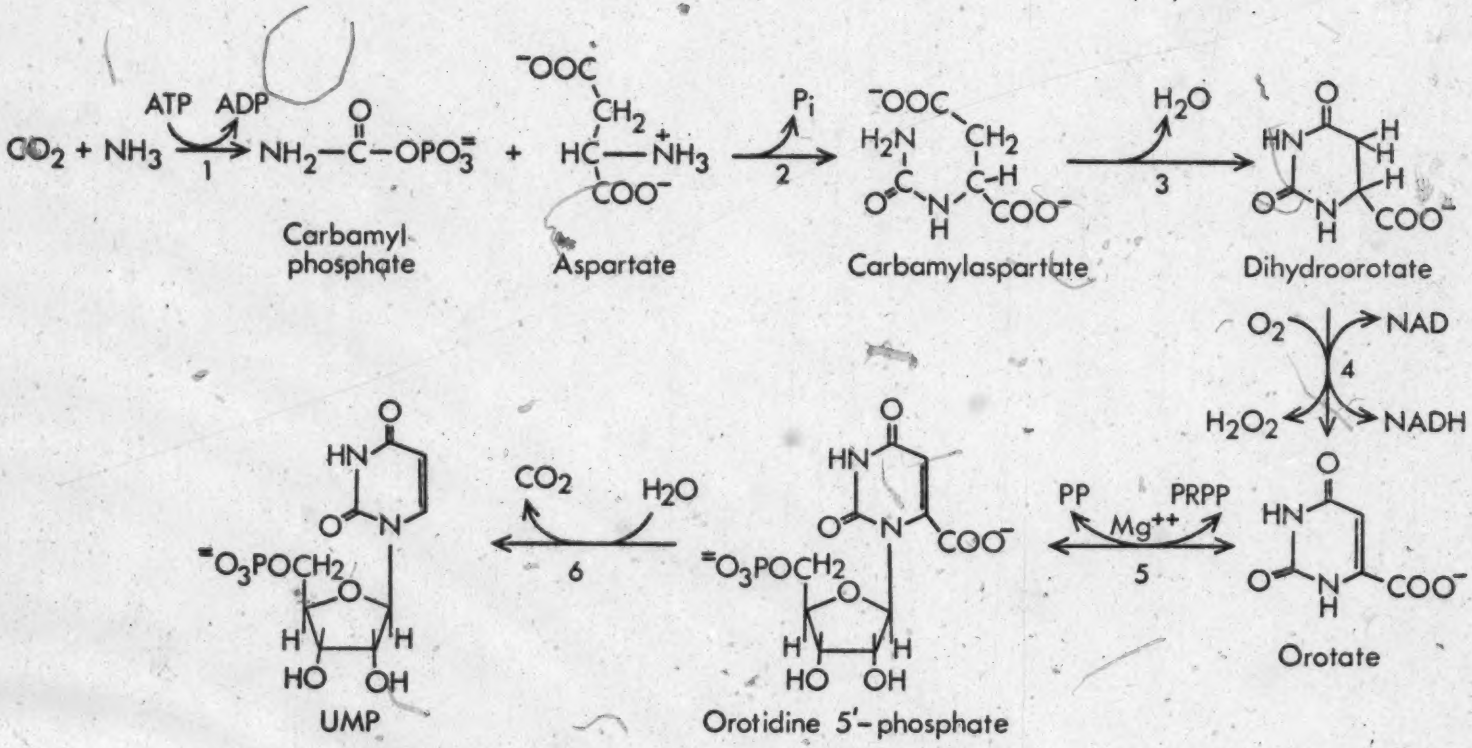


This enzyme was first discovered by Lieberman, Kornberg and Simms (1) from baker's yeast in 1954 during their study of the individual steps in the de novo biosynthesis of pyrimidines. They managed to separate the last two enzymes required for UMP synthesis, OMP pyrophosphonylase and OMP decarboxylase, by ethanol fractionation. Spectral differences for orotidylate and uridylate provided a means of assaying for the decarboxylase where, at pH 8.0 in 20 mM tris, the decarboxylation of one millimolar orotidylate resulted in a decrease of 1.65 absorbance units at 285 nanometers (2). Trapping the radioactive carbon of the carboxylic group attached to the C-6 position of OMP in sodium hydroxide upon release has also provided a sensitive means of assaying OMP decarboxylase.

OMP decarboxylase has been isolated from a number of sources over the last eighteen years. Numerous studies have been done on this enzyme with respect to inhibition of growth of cancer tumors, inhibition of pyrimidine metabolism, and the genetics of the enzyme, since the rare autosomal disease orotic aciduria apparently results from the loss of

Figure 1. Enzymatic steps in the de novo biosynthetic pathway of uridine 5'-monophosphate.

Reaction	Enzyme Involved
1	Carbamylphosphate Synthetase (2.7.2.5)
2	Aspartate Transcarbamylase (2.1,3.2)
3	Dihydroorotase (3.5.2.3)
4	Dihydroorotic Dehydrogenase (1.3.3.1)
5	Orotidylate phosphoribsoyltransferase (2.4.2.10)
6	Orotidylate decarboxylase (4.1.1.23)


ATP
ADP
1
Carbamyl phosphate
Aspartate
Pi
2
Carbamylaspartate
H2O
3
Dihydroorotate
O2
NAD
4
H2O2
NADH
CO2
H2O
6
UMP
Orotidine 5'-phosphate
PP
PRPP
Mg++
5
Orotate

activity of the last two adjacent enzymes in the pyrimidine pathway (see Figure 1).

The properties of the enzyme vary depending on its source. The enzyme from rat liver has been studied by several authors. Blair and co-authors first showed that OMP was involved in de novo pyrimidine biosynthesis in mammals (3). Creasey and Handschumacher purified OMP decarboxylase sixteen-fold with twenty-five per cent recovery (4). Lineweaver-Burk plots (5), based on the rate of release of $^{14}CO_2$ from 7-^{14}C orotidylate, gave a Michaelis constant for orotidylate of 4.5 x 10^{-6} M which compares well with that of 7-8 x 10^{-6} M obtained for yeast. Semi-carbazide, cupric sulfate, barium chloride, sodium azide, sodium fluoride, and hydroxylamine did not inhibit the enzymes from either liver or yeast. Neither magnesiumn or manganese accentuated activity, and ethylenediamine-tetraacetic acid (EDTA) did not decrease activity. Various coenzymes or sulfhydryl activitors failed to stimulate activity. The rat liver enzyme was unstable below pH 6 and stable at pH 8.0 while the yeast enzyme was just the reverse. The pH optima for the two in tris-phosphate buffers were 7.4 and 6.3 respectively. The liver enzyme was unaffected by 30 mM p-chloromercuribenzoate or 1 mM N-ethylmaleimide whereas the yeast enzyme was sensitive to these sulfhydryl reagents, being inhibited 81% by 0.1 mM N-ethylmaleimide and 100% by 0.3 mM p-CMB. The sensitivity of the enzymes to nucleotides is shown in Table I. Detailed kinetic analysis by means of product inhibition of orotidylate decarboxylase has not been reported, though figures implicating a competitive nature of UMP product inhibition have been given by several authors (4, 6).

TABLE I

The Inhibition of Various Orotidylate Decarboxylases by Different Nucleotides

Inhibitor	Yeast K_i (M)	Rat Liver K_i (M)	Cow Brain K_i (M)
UMP	4×10^{-4}	1.5×10^{-4}	2.5×10^{-4}
CMP	7×10^{-4}	30×10^{-4}	1.4×10^{-4}
AMP	8×10^{-4}	inactive	N.D.
GMP	16×10^{-4}	inactive	N.D.
6-Aza-UMP	$7\text{-}8 \times 10^{-7}$	1×10^{-7}	4×10^{-7}
CDP	N.D.	N.D.	8.8×10^{-4}
UDP	N.D.	N.D.	3.0×10^{-3}
CTP	N.D.	N.D.	3.7×10^{-3}

N.D. - not determined.

K_i values were determined on the assumption that competitive inhibition existed. References used were (4, 8) for yeast, (4) for rat liver and (6) for cow brain.

Kasbekar et al. (7) were finally able to separate OMP pyrophosphorylase from OMP decarboxylase by starch gel electrophoresis after a 600-fold co-purification. The pH-activity profile in tris-phosphate buffers showed a sharp peak of decarboxylating activity at pH 6.9. A double reciprocal plot gave a K_m of 2.0×10^{-6} M. They reported that high concentrations of the substrate, orotidine 5'-monophosphate, inhibited enzyme activity. OMP pyrophosphorylase can catalyze the formation of an unusual nucleotide, 5-fluoro-UMP, that inhibited OMP decarboxylase with an inhibition constant of 3×10^{-6} M which closely approximates that seen with 6-azauridine 5'-monophosphate, the most potent inhibitor of Table I. Handschumacher (8) has shown that the strong inhibitory nature of 6-aza-UMP is pH-dependent with an increasing effect seen as the pH was raised above six whereas azauracil, azauridine, and other phosphorylated derivatives of azauridine were non-inhibitory.

Other unusual pyrimidine nucleotides that have shown potent inhibition of orotidylate decarboxylase from various sources include 5-hydroxyuridine 5'-monophosphate (9), 5-azaorotidine 5'-monophosphate, and 5-azauridine 5'-monophosphate (these last two being metabolites of 5-azaorotic acid) (10), and 5-aminouridine 5'-monophosphate (11). These analogues, as well as the two previously mentioned, give relatively strong inhibition when compared to the inhibition of uridine 5'-monophosphate, the product of the reaction. However, this effect could be due to the slower conversion of these unusual pyrimidines nucleotides to higher orders of phosphorylation and incorporation into nucleic acids.

Appel (6) co-purified the enzymes OMP pyrophosphorylase and OMP

decarboxylase 600-fold to apparent homogeneity by acrylamide gel electrophoresis. The latter enzyme was stable to storage at -15° C while the former was not. The K_m for OMP for the decarboxylase from this source was 3.0×10^{-6} M. Table I reveals the effectiveness of various nucleotides as inhibitors. Heat denaturation studies at 55° revealed an exponential decay of activity with concentrated amounts of enzyme and a linear decay with lesser amounts. UMP, CMP, and 6-aza-UMP were able to stabilize OMP decarboxylase under these heat conditions.

The presence of OMP decarboxylase has also been shown in Neurospora crassa (12), in plants (13, 14), in chicks (15), and in man (16, 17).

Thus it is now recognized that the de novo synthesis of uridine 5'-monophosphate occurs by the pathway outlined in Figure I.

The control of pyrimidine metabolism appears to be complex. In Saccharomyces cerevisiae, the overall control of pyrimidine synthesis is feedback inhibition by UTP or CTP on aspartate transcarbamylase and carbamylphosphate synthetase. In addition to end product inhibition on the various enzymes of the pathway, intermediates of the pathway appear to induce the synthesis of enzymes found later in the pathway (18). When various E. coli mutants with genetic blocks at different points in the pathway to UMP are starved for pyrimidines, induction of enzyme synthesis occurs on both sides of the genetic block (19). Synthesis of the last four E. coli enzymes appears to be coordinately linked, even though the enzymes are not all together on the chromosome (20, 21).

In Saccharomyces cerevisiae, Lacroute (22) showed that the pyrimidine biosynthesis depends on enzyme repression and induction as

well as feedback inhibition. The last three enzymes appear to be induced by dihydroorotic acid. However, such induction studies are performed with difficulty since the intermediates ureidosuccinic acid, dihydroorotic acid, and orotic acid are not significantly incorporated by yeast, whereas uracil, uridine, and cytosine are all actively transported by specific pyrimidine permeases (23) and are then subsequently converted to UMP by cytosine deaminase or uridine ribohydrolase and UMP phosphatase. The last four enzymes are not regulated by end product repression whereas those of E. coli are. Heterozygotes of yeast with only one-half the activity for any one of the pathway's biosynthetic enzymes grew as rapidly as did the wild type yeast suggesting that pyrimidine biosynthesis in yeast is controlled by enzyme induction, since there appear to be ample amounts of each present. The genes for the later enzymes in the synthesis of UMP are not linked. However, the first two enzymes, carbamylphosphate synthetase and asparate transcarbamylase, are simultaneously controlled by feedback inhibition and repression and have been shown, by co-purification, to exist in an enzyme complex, the stability and properties of which depend on the presence of UTP (24). Recently, it has been reported that the rare human disease, orotic aciduria, may not be due to a genetic alteration of two adjacent enzymes but rather to an abnormal complex of OMP phosphoribosyltransferase and OMP decarboxylase (25), both of which are substrate-induced as in the yeast system.

Due to the variations in the control of de novo pyrimidine biosynthesis, it would appear that the complete description of the properties of each enzyme will be dependent on the source from which it is isolated.

The purpose of this thesis will be to study aspects of the yeast OMP decarboxylase enzyme: its reaction mechanism as revealed by initial rate kinetics, product inhibition and radioisotope studies; its peculiar sensitivity to sulfhydryl reagents; and some of its other properties.

II. MATERIALS

Materials used in the course of this study were of the highest purity available. UMP, orotic acid, orotidine, disodium EDTA, N-ethylmaleimide, iodoacetamide, and phosphocellulose were obtained from the Sigma Chemical Company. Inorganic salts or acids were obtained from either J. T. Baker or Fisher Scientific. Sephadex G-25 and G-100 (40-120u) were obtained from Pharmacia. Protamine sulfate (salmon sperm) was purchased from Pierce Chemical Company; and "ultrapure" ammonium sulfate was from Mann Research Laboratories. Phosphoribosylpyrophosphate was purchased from P. L. Biochemicals. OMP was obtained from either Calbiochem or P. L. Biochemicals.

Fleischmann's baker's yeast (Saccharomyces cerevisiae), the source for the enzyme, was obtained from a local distributing firm.

III. SYNTHESIS OF OROTIDINE 5'-MONOPHOSPHATE

During the initial stages of this study, orotidylic acid could not be purchased commercially. OMP was synthesized from orotidine using phosphorylchloride in a trialkylphosphate solvent system. This novel method of synthesis, which gives preferential phosphorylation at the 5' position of the nucleoside used, was developed by Yoshikawa and Kato (26, 27) and by Imai et al. (28). The method was first used by these two groups to obtain the more commonly found 5' nucleotides IMP, GMP, AMP, UMP, and CMP. Yields of 70 to 100 per cent were reported for the 5' nucleotide (higher yields were obtained when 2'3'-O-isopropylidene nucleosides were used).

The reaction mixture for OMP synthesis contained:

Orotidine	-	0.33 millimoles
Phosphorylchloride	-	4.4 millimoles
Double distilled H_2O	-	2 millimoles
Triethylphosphate	-	19.6 millimoles

The reaction was started by rapidly adding the phosphorylchloride dropwise in a fumehood, and was carried out for twenty-two hours at 2 to 4° C in a stoppered tube with continuous stirring.

The anticipated white dichloronucleotide precipitate did not form with the addition of anhydrous ether. The ether was removed by flash evaporation leaving a residual yellow oil.

An anion exchange column (AG-2-x8, 50-100 mesh, chloride form) of 35 ml total volume was prepared by washing with 0.05 N HCl until the optical density of the eluant at 260 nm was zero. The column was then washed with water until the pH rose to five.

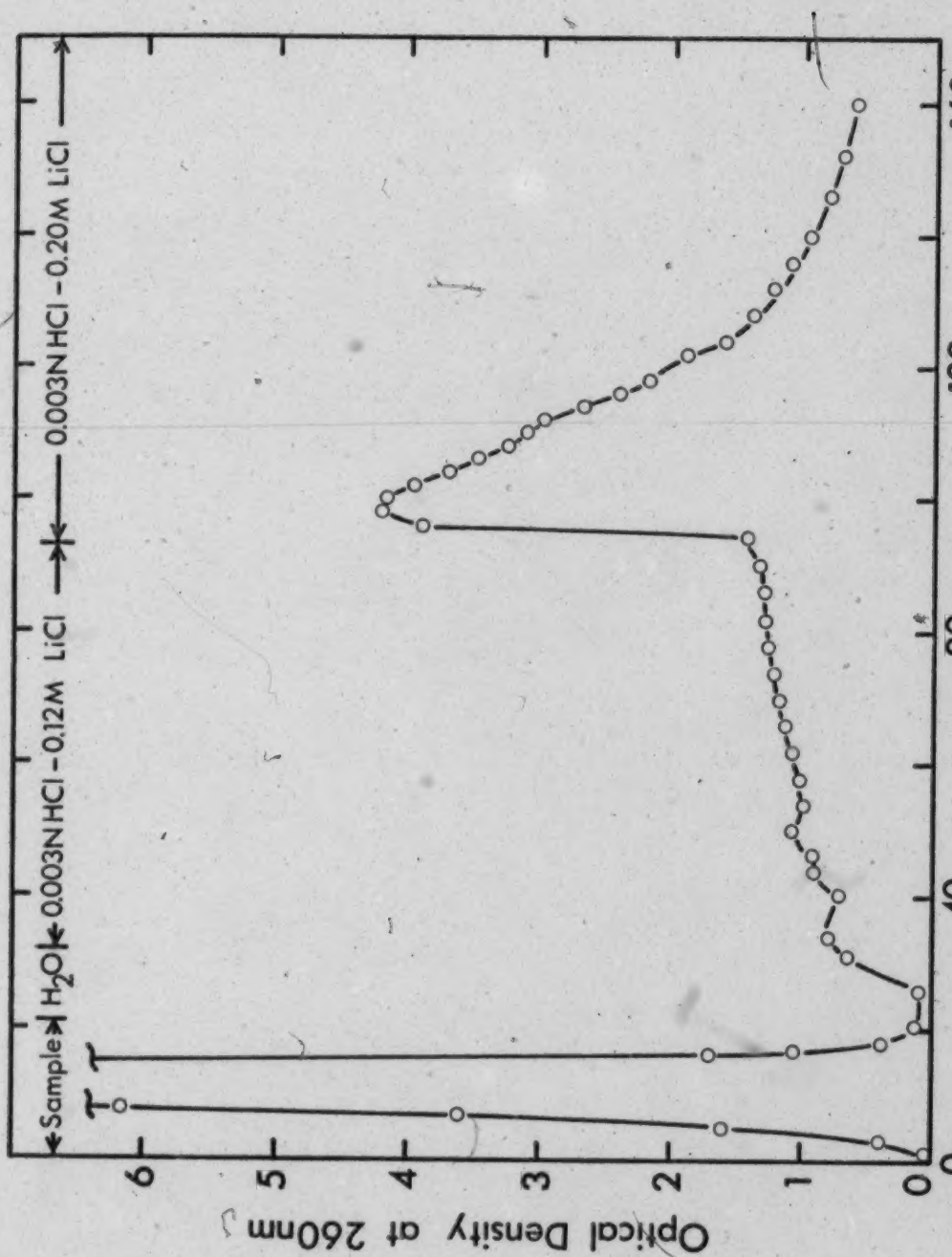
The oil was diluted to 25 ml with water and passed onto the column. Elution from the column with various solvents was followed spectrophotometrically at 260 nm as shown in Figure 2.

Ultraviolet spectral analysis of the eluant could not reveal whether orotidine or orotidine 5'-phosphate was the ultraviolet absorbing compound present. Therefore, crude yeast extracts were added to blank solutions and to aliquots of selected eluant fractions and the spectral changes were observed after two and one-half hours. Shifts in the peak maximum from 266 nm to 262 nm for fractions 95 through 121 indicated the presence of orotidine 5' phosphate, since the breakdown of orotidine to orotic acid would have given a different initial spectrum, with a peak maximum nearer to 277 nm (29) and orotidine itself is believed to be metabolically inert.

Fractions 95 to 121 of Figure 2 were lyophilized to dryness and taken up in 8 ml of water. The lithium chloride was removed by passing the OMP-LiCl solution through a diethyl cellulose A23 column (previously washed with 0.01 N HCl, water, 0.01 N NaOH, and water) and an equal volume of effluent was collected and checked for ultraviolet absorbance to measure the binding to the column. The column was washed with water until the effluent tubes contained no chloride ions detectable by 1% $AgNO_3$ addition, and then the column was washed with 1M triethylammonium bicarbonate until the optical density at 265 nm equalled zero. Recovery of material initially bound was 85%.

Fractions showing optical density at 265 nm were pooled in a 500 ml round-bottomed flask. Fifty per cent methanol was added and the solution was then flash evaporated. This was repeated until the triethylammonium bicarbonate odor was gone. The residue was then taken up in

Figure 2: Isolation of a phosphorylated derivative of orotidine by anion exchange chromatography on AG-2-X8. Absorbance at 260 nm is plotted against the fraction number. Six milliliter fractions were collected with the flow rate set at 20 ml/hr. The column was eluted with 66 ml of double distilled water, 390 ml of 0.003 N HCl - 0.12 M LiCl, and 620 ml of 0.003 N HCl - 0.20 M LiCl after the initial 25 ml sample was applied.


Sample
H2O
0.003N HCl - 0.12M LiCl
0.003N HCl - 0.20M LiCl
Optical Density at 260nm
Fraction Number
0
1
2
3
4
5
6
40
80
120
160

water and a recovery of 58 umoles was obtained. The solution possessed a deep yellow color.

This solution was used for assaying orotidylate decarboxylase activity during the development of a purification procedure in the early stages of this work. Commercial OMP replaced it when it became available.

IV. PURIFICATION OF OROTIDYLATE DECARBOXYLASE

The enzyme orotidylate decarboxylase has been purified from a variety of sources to various degrees (4, 6, 7, 12).

The enzyme source in this study was baker's yeast (Saccharomyces cerevisiae), selected because of:

1. its commercial availability,
2. its use by other authors in the study of OMP decarboxylase thereby allowing comparison with previously published results,
3. it is the source from which the original discovery of the enzyme by Lieberman et al. was made.

Since five pounds of yeast yield approximately 167 International Units of orotidylate decarboxylating activity, it appeared unnecessary to seek unusual sources with elevated levels of this enzyme or to provide a controlled nutrient broth for growth to induce OMP decarboxylase.

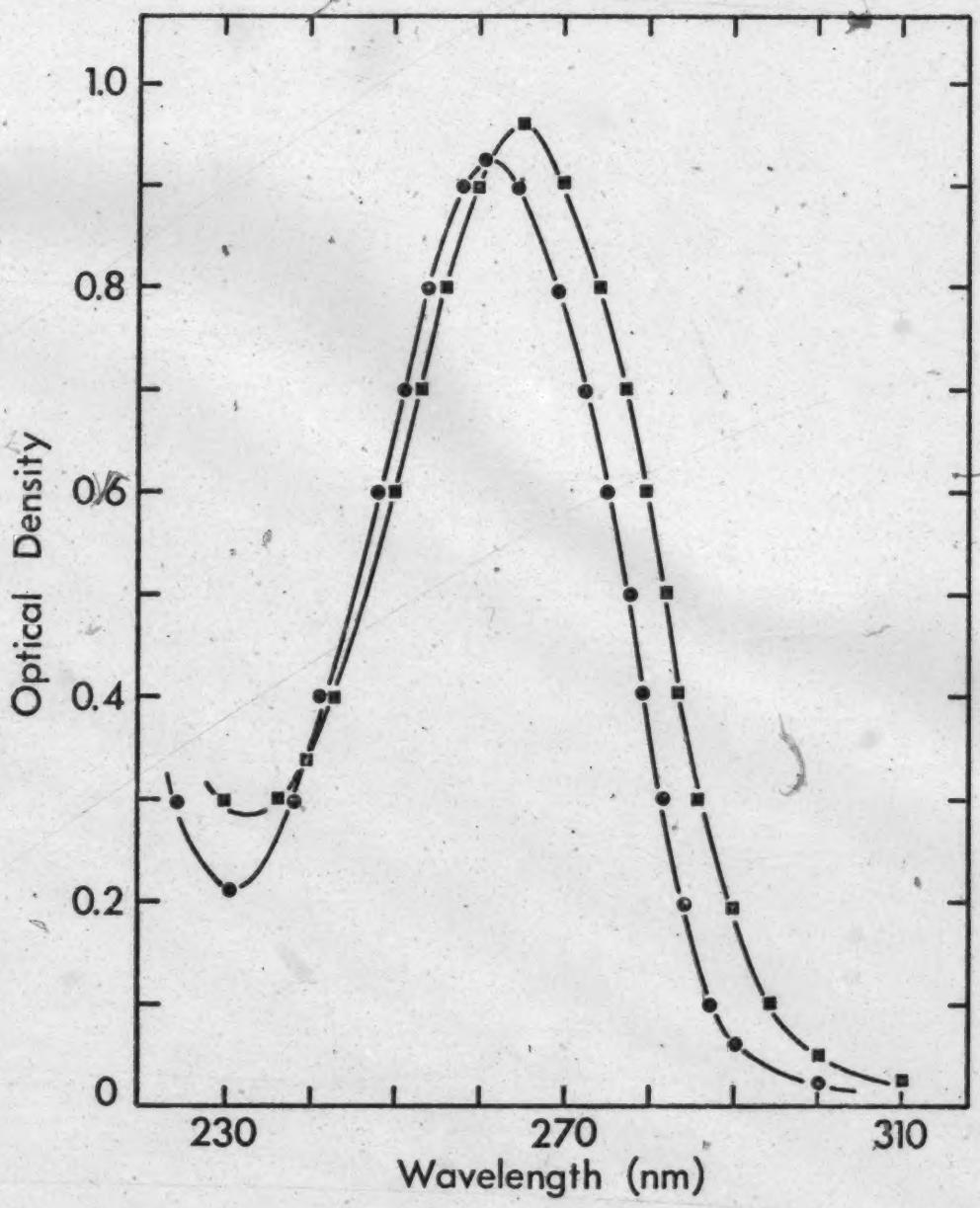
A. Assay Procedure

The spectrophotometric assay used was based on the decrease in optical density (maximal at 285 nm) as OMP is decarboxylated to UMP as in Figure 3. Lieberman, Kornberg and Simms (2) reported that at this wavelength the change in molar extinction coefficients was -1650 at pH 8.0. In our assay medium at pH 7.0, the molar extinction coefficient change calculated from Figure 3 was -1930 at 30° C. The assays were followed continuously on either a Cary model 15 or on a Gilford 240 (spectrophotometer). These assay solutions contained;

85 mM potassium phosphate - 5 mM EDTA, pH 7.00	-	1000 ul
10 mM OMP	-	10 ul
Enzyme fraction for testing	-	10 to 50 ul

The blank solution contained water in place of OMP. Assays were started

Figure 3: Spectra of the initial and final products of the reaction catalyzed by OMP decarboxylase using 0.0924 umoles of OMP. Spectrum of OMP (■). Spectrum of UMP (●). At pH 7.00 the millimolar extinction coefficients of OMP and UMP are 10.40 and 10.00 respectively. With these values 100% conversion of OMP to UMP is observed spectrally. Thus the millimolar extinction coefficient change at 285 nm equals $\frac{0.182 - 0.360}{0.0924}$ or -1.93.


1.0
0.8
0.6
0.4
0.2
0
Optical Density
230
270
310
Wavelength (nm)

with the addition of OMP to the one centimeter light path sample. Under these conditions, the assays were linear over the first ten minutes. A unit of enzyme activity, by definition, decarboxylated one umole of OMP per minute at 30° C. Specific activity was expressed as units per milligram of protein.

Protein determinations on the various aliquots sampled throughout the purification were performed in two ways. Rough estimates of the protein present were calculated from the absorbances at 280 and 260 nm (30). More accurate measurements of the protein concentration were based on the method developed by Lowry et al. (31) using bovine serum albumin as the reference standard.

B. Homogenization

Five pounds of baker's yeast were crumbled into one liter of 0.05 M potassium phosphate (KP) - 1 mM EDTA, pH 6.8, in a four liter beaker at 0° C, mixed well with a stirring rod and left standing for 30 to 60 minutes at 0° C. The slurry was then poured into a Gifford-Wood Micromill which had been previously chilled to -20° by circulating coolant. Glass beads, of 0.2 mm diameter, to a total weight of 1.5 kilograms were slowly added and the gap setting adjusted to 40. Homogenization resulted from running the mill for a total of 30 minutes at 100 volts with intermittent cooling periods at 40 volts to keep the temperature of the mixture below 10°.

The contents of the mill were then run off at a rheostat setting of 40 volts (after allowing the temperature of the mill's chamber to cool to 0° C) into a four liter beaker and left for 45 minutes to permit the glass beads to settle out. The solution above the beads was

then decanted into centrifuge bottles. The bottles were centrifuged at 13200 x g for 35 minutes at 0°. The supernatant was aspirated off into a two liter flask, cooled in ice, and an aliquot termed "Assay Fraction 1" was saved.

The beads could be washed with one liter of the buffer used above and the washing pooled with the initial extract if desired.

C. Protamine Sulfate Treatment

Protamine sulfate (0.5 g per 100 ml of the supernatant extract) was added slowly and stirred for 60 to 90 minutes at 0°. The solution was then centrifuged for one hour at 9000 rpm in the GSA rotor. The supernatant solution was then centrifuged for 10 to 18 hours at 19000 rpm at 1° in a #19 rotor of a Spinco L2-65B ultracentrifuge. The lipid layer was first removed and then the supernatant was aspirated off into a flask cooled in ice. An aliquot (termed "Assay Fraction 2") was saved.

D. Ammonium Sulfate Fractionation (64-81%)

NH_4OH was added to the supernatant to raise the pH from 5.8 to 6.2. Then $(NH_4)_2SO_4$ (40 grams per 100 ml) was added slowly over 20 to 30 minutes, with additions of 1.0 M NH_4OH to maintain a pH of 6.2. The solution was stirred for one hour at 0°. The solution was centrifuged for 50 minutes at 13000 x g. The supernatant was aspirated off and an aliquot saved ("Assay Fraction 3"). $(NH_4)_2SO_4$ (15 grams per 100 ml) was added to the supernatant and the solution stirred for 40 minutes at 0°, followed by centrifugation for 1 hour at 13000 x g. The supernatant was aspirated off and an aliquot was saved ("Assay Fraction 4").

The precipitate was dissolved in a minimal volume of 20 mM KP - 1 mM EDTA, pH 6.2 and an aliquot saved ("Assay Fraction 5").

E. Desalting

The dissolved protein solution was then immediately passed onto a Sephadex G-25 (medium) column of 290 ml (25 mm dia. x 600 mm) with a flow rate of 60 to 80 ml per hour, and was eluted with 20 mM KP - 1 mM EDTA, pH 6.2 buffer. 10.2 ml fractions were collected and the protein-containing fractions were assayed for OMP decarboxylase activity. The fractions containing the enzyme were pooled (150-200 ml) and concentrated to 20 to 30 ml through an Amicon Ultrafiltration cell apparatus using the model 52 cell and a UM 20E membrane. Several dilutions of this concentrated protein with buffer (and subsequent concentration in the cell apparatus) ensured the complete removal of ammonium sulfate contamination. A small aliquot termed "Assay Fraction 6" was saved.

F. Phosphocellulose Chromatography

Substantial purification was achieved by the selective elution of UMP decarboxylase from phosphocellulose by the product of its reaction, UMP.

The concentrated sample was passed into a phosphocellulose column of 400 to 500 ml bed volume (2.5 x 100 cm). Elution was begun with one bed volume of the starting buffer (20 mM KP - 1 mM EDTA, pH 6.2), followed by two or three bed volumes of 20 mM KP - 1 mM EDTA - 2 mM UMP, pH 6.2 and three bed volumes of 300 mM KP - 1 mM EDTA, pH 6.2. Using a flow rate of 10 to 15 ml/hr, 9 ml fractions were collected and were assayed for enzyme activity. (The dilution of UMP upon addition to the assay solution obviated any significant inhibitory effect.) Fractions containing the UMP decarboxylase activity were pooled and concentrated to 6 - 10 ml using a Model 12 ultrafiltration cell and a UM 20E membrane. This sample or an

Figure 4: A typical elution profile of OMP decarboxylase from a 2.5 cm x 93 cm phosphocellulose column. The flow rate was 10 - 15 ml/hr. 9.1 ml fractions were collected. Enzyme activity assays used followed conditions outlined under Assay Procedure. OD_{280} were taken in the Gilford 240 using 20 mM KH_2PO_4 - 1 mM EDTA, pH 6.2 as the reference blank. Elution was performed with 20 mM KP - 1 mM EDTA, pH 6.2 (A) and 20 mM KP - 1 mM EDTA - 2 mM UMP, pH 6.2 (B).

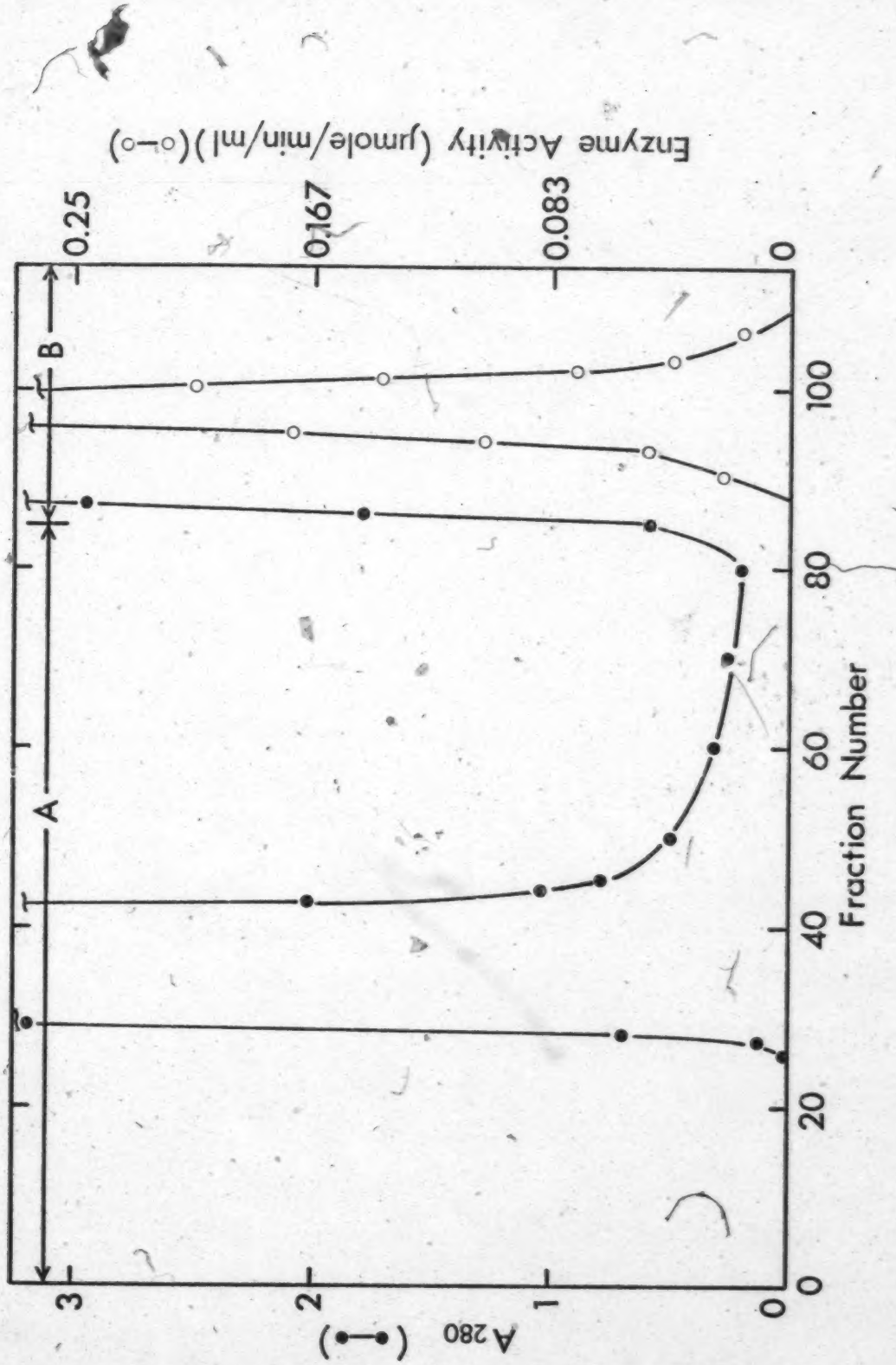
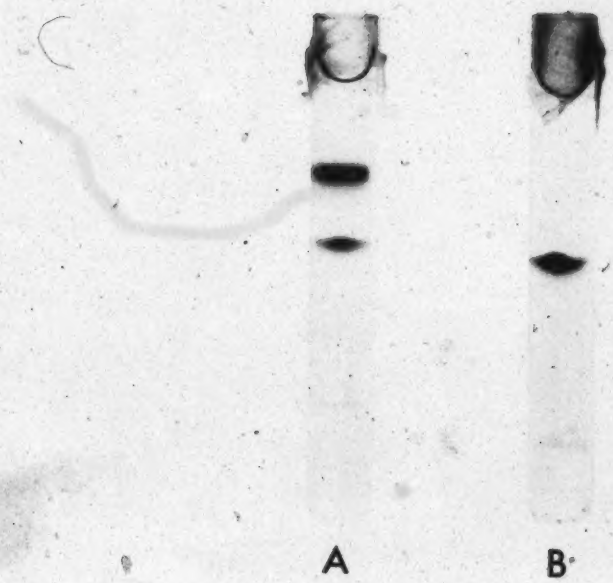

Enzyme Activity (μmole/min/ml) (o—o)
0.25
0.167
0.083
0
B
A
3
2
1
0
A280 (•—•)
0
20
40
60
80
100
Fraction Number

Figure 5: Analysis of protein homogeneity by polyacrylamide gel electrophoresis.

A. 6.2 ug of phosphocellulose OMP decarboxylase applied to a 7% gel and subjected to electrophoresis for 3 - 3-1/2 hours at 3-1/2 ma per gel. Gel and sample preparation followed the method outlined by Ornstein (33).

B. 6 ug of Sephadex G-100 OMP decarboxylase applied to a 7% gel. The procedure followed was outlined in part A.

Staining was accomplished with Coomassie blue.


A
B

aliquot thereof was termed "Assay Fraction 7".

A typical phosphocellulose elution profile pattern is illustrated in Figure 4. Most of the protein passes directly through the cationic exchanger while OMP decarboxylase is tightly bound. The use of a substrate or substrate analog as an eluting device was first suggested by Koshland et al. (32). In the present case, the addition of the reaction product, UMP, releases the enzyme from the column, presumably by virtue of the increased negative charge of the E·UMP complex. Recovery of applied activity was 85-100% per cent.

During repeated runs with the phosphocellulose column, a visible indicator of probable success was the appearance of a red band at the top of the column that could be eluted only with high salt concentrations. The absence of this band almost invariably was indicative of protein overloading or other problems and no resolution of OMP decarboxylase from the bulk of the protein was seen. The phosphocellulose columns worked best when the length to width ratio of the column was greater than 30 to 1 and when the sample was completely void of $(NH_4)_2SO_4$ and was applied in a volume less than 5% of the total column volume.

As can be seen in Figure 5A, OMP decarboxylase preparations following this step are not homogeneous, having two protein bands detectable by electrophoresis in 7% polyacrylamide gel.

G. Sephadex G-100 Chromatography

The enzyme from the previous step could be passed through a Sephadex G-100 column (1.0 cm x 64 cm) and eluted with 50 mM KP, pH 7.0. The fractions with decarboxylating activity could be concentrated by ultrafiltration through a UM 20E membrane to give "Assay Fraction 8".

TABLE II

Purification Summary of Orotidylate Decarboxylase

Step	Fraction	Volume (ml)	Total Protein (mg)[a]	Total Activity[b]	Specific Activity[c]	Purification (fold)	Recovery (%)
1	Homogenization	1100	8690				
2	Protamine sulfate and high speed spin	810	6642	161	.024	1	100
3	0-64% ammonium sulfate saturation	860	8153	137.5	N.D.	N.D.	85.6
4	64-85% ammonium sulfate supernatant	900	1683	27.0	.016	0.67	16.9
4	Dissolved precipitate	53.5	656.5	102	0.157	6.44	63.6
5	Desalting and phosphocellulose chromatography	11	8.8	27.5*	3.125	129	45.4
6	Sephadex G-100 column chromatography**	11.7	0.16	0.56	3.40		12.3

a - Determined by the Lowry method.
b - Expressed in terms of umoles decarboxylated per minute.
c - Expressed as umoles OMP decarboxylated/minute/mg. protein.

*Only 38.5 umoles/min of Step 4 was applied.

**This is based on a later preparation using phosphocellulose OMP decarboxylase of 3.40 umoles/minute/mg. protein where one milliliter of 620 ug/ml. was applied.

This step was not routinely used since the enzyme sample at the end of the phosphocellulose step was of sufficient purity for this study.

Passage of the OMP decarboxylase through a Sephadex G-100 column resulted in a homogeneous protein (Figure 5B), though the recovery of only 27% of the activity applied (based on two column runs) was less than anticipated.

Though there appeared to be no further purification by this step, made evident by increased specific activity, the loss of one protein band (see Figure 5) shows that removal of a contaminant had indeed taken place. The lower recovery of activity in the 50 mM potassium phosphate, pH 7.0 buffer implies that some inactivation of the protein has probably also occurred. Lowering the pH of the eluting buffer might stabilize the enzyme increasing the yield and giving a truer measure of the purification. This was not tried in the present study.

The overall result of this five step procedure is a substantial purification of OMP decarboxylase with adequate recoveries. Table II gives a summary of the results from such a purification run. The simplicity of this method should make possible the study of this enzyme under more controlled conditions than has been previously applied to OMP decarboxylase from yeast. Only steps one though five were actually applied in all purifications. Step 6 was applied to part of one preparation for certain experiments.

V. PROPERTIES OF OROTIDYLATE DECARBOXYLASE

The study of a particular enzyme brings out many of that enzyme's pecularities. Before kinetic detail, reaction mechanisms, or cellular control processes can be explained, the most favourable conditions for enzyme activity must be determined.

A. Lack of a Demonstrable Metal Requirement

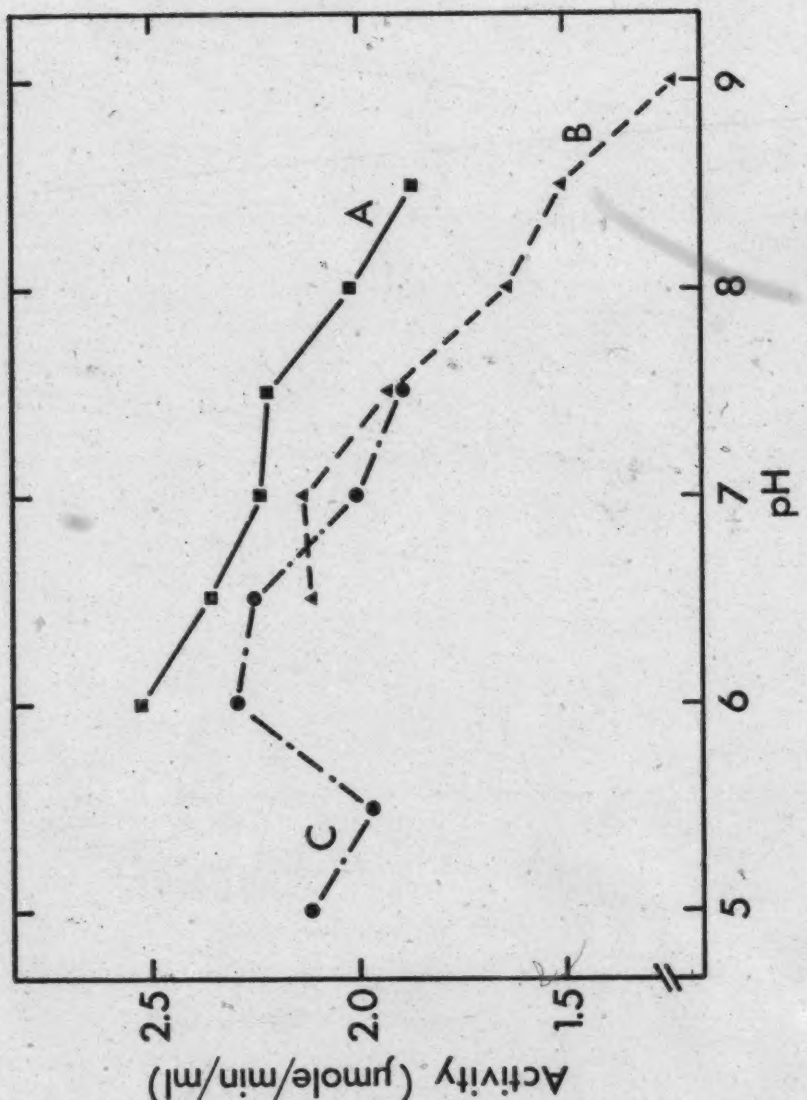
Many enzymes require the presence of a divalent cation as an essential component of the reaction, either as a prosthetic group on the enzyme or as a substrate complexing agent to give a more favourable form of the substrate for binding to the enzyme. In the case of OMP decarboxylase, 0 to 20 mM concentrations of $MgCl_2$ did not stimulate the reaction. EDTA, however, stimulated the reaction 15 to 20 per cent over that of the control in the 5 to 20 mM concentration range tested. Consequently, EDTA, at a concentration of 5 mM, was present in all assays performed.

B. Effect of pH and Buffer Composition on Activity and Stability

Figure 6 reveals the pH profile of OMP decarboxylase in three different buffer systems tested. Solutions were prepared within 1.5 pH units of the pK of the buffers used. Two of these buffers are ones commonly used in enzymatic assay systems. The third one, sodium cacodylate ($(CH_3)_2As(O)O^-Na^+$), was tested since the pK_a of this buffer is close to the pH optimum previously reported for this enzyme (4).

Since the activities were consistently higher for the phosphate buffer system over its pH range, this buffer system was selected for use experimentally.

Figure 6: Activity of OMP decarboxylase as a function of pH. The activity is expressed as umoles of OMP decarboxylated per minute per milliliter protein, using 0.10 mM OMP in a 1 ml cuvette. After a 15 minute enzyme preincubation period at the selected pH, assays were initiated by OMP addition and followed spectrophotometrically at 285 nm. (A) 50 mM potassium phosphate (pK_a = 7.20); (B) 50 mM Tris-Cl (pK_a = 8.3); (C) Sodium cacodylate (pK_a = 6.19).


Activity (μmole/min/ml)
2.5
2.0
1.5
5
6
7
8
9
pH
A
B
C

The effect of buffer concentration was tested at pH 6.0 where the activity appeared highest. After a fifteen minute pre-incubation period, activity rose as the phosphate concentration was raised to 50 mM. The activity then leveled off until the phosphate concentration reached 100 mM after which it started to decrease. An intermediate concentration on the activity plateau, 80-85 mM, was chosen for standard use. At room temperature the enzyme is quite stable in 80 mM potassium phosphate (KP), pH 6.10. The enzyme loses 50% of its activity after incubation at room temperature for about 8 hours in this buffer at a protein concentration of 16 ug/ml.

C. Storage Procedures and Enzyme Stability

Lyophilization in the presence of dithiothreitol was the standard storage procedure used by Creasey and Handschumacher (4). However, after freeze-drying OMP decarboxylase overnight and immediately redissolving the residue, there was a 60% loss of decarboxylating activity in the absence of dithiothreitol and a 40% loss with 0.1 mM dithiothreitol included.

Storage of the phosphocellulose elutant protein at concentrations of less than 1 mg/ml in 20 mM KP - 1 mM EDTA, pH 6.20 at -20° resulted in very little loss of activity over several months (30% over a five-month period with repeated freezing and thawing).

OMP decarboxylase, desalted by Sephadex G-25 column chromatography, was also stable to freezing and thawing in 20 mM KP - 1 mM EDTA, pH 6.20 with a six-month period required before the decay of activity reached 50% of the initial activity. Addition of ethylene glycol (33% v/v) to prevent the protein solution from freezing at -20° resulted in a similar retention of activity. OMP decarboxylase from yeast thus appears to be

quite stable and can be stored in a variety of ways. The enzyme used for studies, the fraction from phosphocellulose, was routinely stored at -20° C.

D. Proportionality of Reaction Velocity to Enzyme Concentration

The initial rate of decarboxylation of 55 nanomoles of OMP, in a 1 ml reaction assay, was shown to be proportional to the protein concentration from 0.1 to 5.0 ug/assay.

E. Molecular Weight

No molecular weight for this enzyme has yet been reported in the literature, apparently because the enzyme has not previously been purified to homogeneity. OMP decarboxylase eluted by UMP from a phosphocellulose column gave only two protein bands following disc electrophoresis (see Figure 5A, Chapter IV). Passage of the enzyme through a Sephadex G-100 column using 50 mM KP, pH 7.00 as the eluting buffer gave an elution volume (V_e) to void volume (V_o) ratio of 1.26 and 1.28 for two successive runs. From a plot of the V_e/V_o ratio determined for proteins of known molecular weight against the logarithm of that weight as shown in Figure 7, a molecular weight of 153,000 $\pm$ 5,000 was obtained for OMP decarboxylase. The experimental details for this experiment are given in the legend to the figure.

Fractions from the Sephadex G-100 column were concentrated by evaporation in vacuo (for 18 hours with the aid of silica desiccant). Polyacrylamide gel electrophoresis of 6 micrograms of the protein revealed only one band, with a mobility similar to the faster of the two bands seen with the phosphocellulose-purified OMP decarboxylase. Application of the procedure of Weber and Osborn (34) for the determination

Figure 7: Determination of the molecular weight of OMP decarboxylase by Sephadex G-100 column chromatography. The V_e/V_o of standards are averages of three runs. The V_o was determined with the use of 0.2% Blue Dextran. Molecular weights of the protein standards were: 13,800 for ribonuclease; 17,000 for myoglobin; 45,000 for ovalbumin, 64,500 for rabbit hemoglobin; 67,800 for bovine serum albumin; and 142,000 for *E. coli* succinyl CoA synthetase.

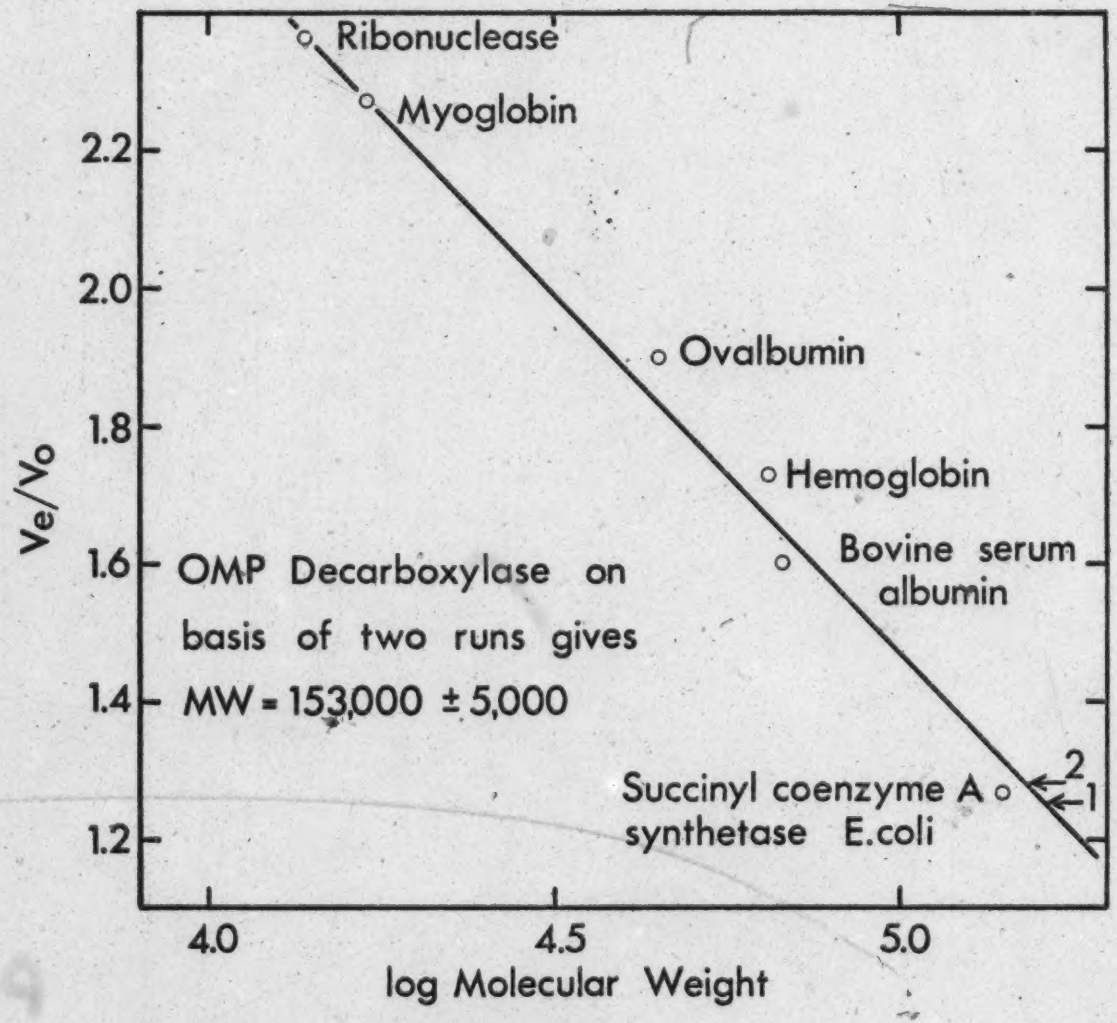
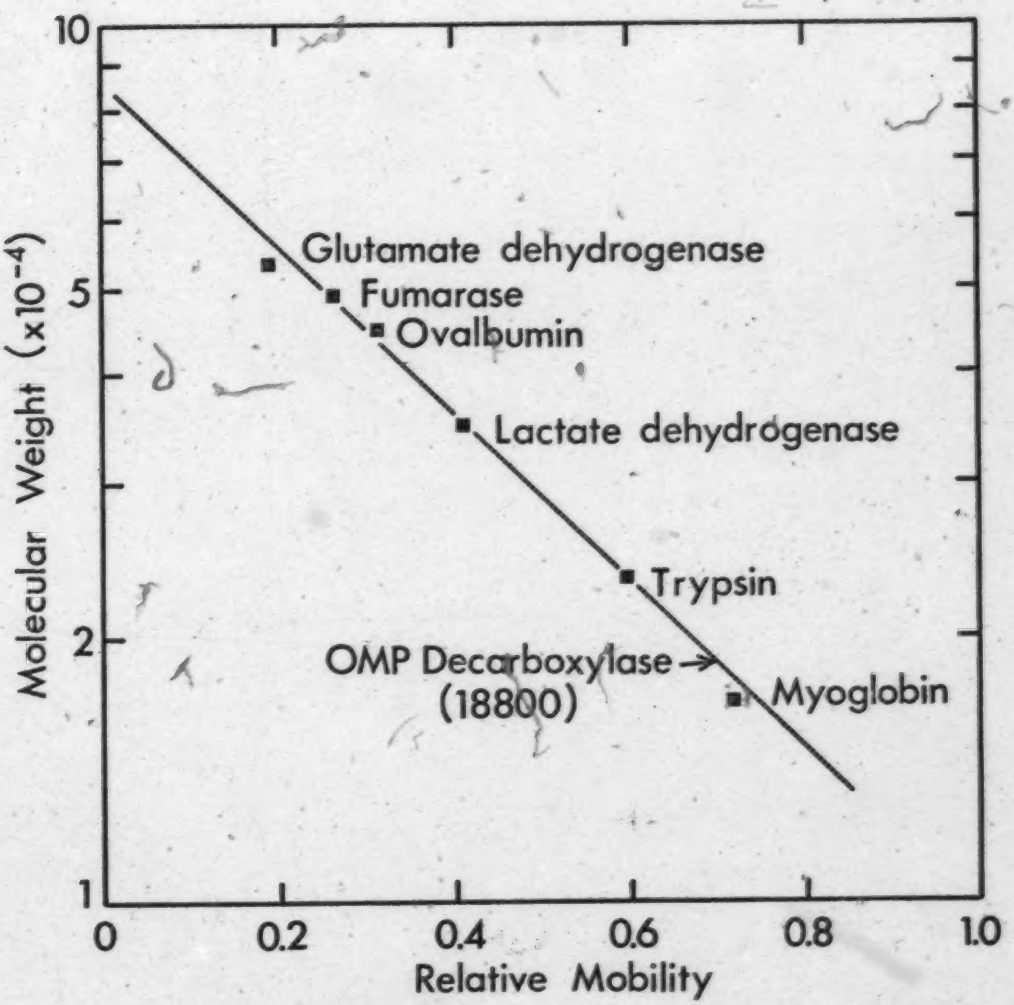

Ribonuclease
Myoglobin
Ovalbumin
Hemoglobin
Bovine serum albumin
Succinyl coenzyme A synthetase E.coli
2
1
OMP Decarboxylase on basis of two runs gives MW = 153,000 ± 5,000
Ve/Vo
2.2
2.0
1.8
1.6
1.4
1.2
4.0
4.5
5.0
log Molecular Weight

Figure 8: Determination of the molecular weight of OMP decarboxylase subunits. Relative mobilities of proteins given are the average of three separate determinations. Samples applied were composed of 25 or 50 ul of dissociated protein solution, 3 ul 0.05% bromophenol blue, 50 ul glycerol, 5 ul 2-mercaptoethanol, 25 ul gel buffer, and 25 ul water. This entire mixture was applied to 0.6 x 9 cm polyacrylamide gels.


10
5
2
1
Molecular Weight (x10⁻⁴)
Glutamate dehydrogenase
Fumarase
Ovalbumin
Lactate dehydrogenase
Trypsin
OMP Decarboxylase →
(18800)
Myoglobin
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility

of the molecular weights of protein subunits in sodium dodecyl sulfate revealed an average mobility of 0.697 $\pm$ 0.004 based on the results of three gels. From a plot of the logarithm of the subunit molecular weight versus the mobility relative to that of a tracker dye (bromophenol blue) as shown in Figure 8, the subunit molecular weight was calculated to be 18,800. The protein mobility was based on densitometer tracings at 540 nm with corrections made for gel expansion in the 7% acetic acid.

Thus OMP decarboxylase appears to be a protein of molecular weight near 153,000, perhaps composed of eight identical subunits.

VI. INITIAL VELOCITY STUDIES OF THE OMP DECARBOXYLASE REACTION

A. Introduction

The study of the kinetics of an enzyme provides a means of studying how a reaction may proceed. It can not prove, however, that a mechanism is correct but can rule out alternatives which are not consistent with the experimental data. Kinetics as such is a limited tool in the study of the reaction mechanism of an enzyme. By initial velocity studies, one can not determine the number of intermediate complexes that intervene before the release of products (35). Kinetic measurements can, however, shed light on the order of combination of substrates and release of products. The methods devised by Cleland (36, 37, 38) have been very helpful in the systematic kinetic examination of reaction sequences.

No detailed kinetic study of OMP decarboxylase has appeared in the literature though reports bordering on this area have appeared. Several studies with OMP decarboxylase, however, have dealt with the use of nucleotide inhibitors as a means of limiting de novo pyrimidine biosynthesis (8, 9, 10, 11, 39).

It is hoped that the results of this study will help lead to the elucidation of this reaction mechanism.

B. Methods

Initial velocity studies were carried out in Gilford 240 and Cary 15 spectrophotometers by measuring the rate of change of absorbance at 285 nm. Velocities were determined from the initial slope of the reaction progress curve using a millimolar extinction coefficient change

of -1.93 for OMP decarboxylation to UMP. Initial velocities were expressed as umoles OMP decarboxylated per minute per milliliter of protein. Each milliliter of protein contained approximately 250 ug that yielded 2 bands on gel electrophoresis. Concentrations of substrates and inhibitors which varied with the experiments performed will be given in the text or figures.

Estimates of the Michaelis constant (K_m) and maximum velocity (V) from the available data were obtained in two ways:

1. By application of an unweighted linear regression analysis (Olivetti program #681009) to the reciprocals of both the velocities and the OMP concentrations. Standard errors of the intercept (1/V) which were less than 15% of the intercept value were taken as indicative of adherence to the Lineweaver-Burk equation. The error in the slope (K_m/V) is not given by this program.

2. By weighted fits to the Lineweaver-Burk equation as developed by Wilkinson (40). The Olivetti computer program developed for this gives a corrected K_m and V together with their respective standard errors, on the assumption that the data will fit the equation obtained by Lineweaver and Burk (5). In this case the slope was taken as K_m/V with the error in the slope being equal to the sum of the percentage errors of Michaelis constant and maximum velocity respectively.

C. Results

1. Initial Rate Studies with OMP

The mean maximum velocity and Michaelis constant for OMP from five separate experiments were 1.40 ± 0.04 umoles OMP decarboxylated/min/ml protein and 5.1 ± 1.5 uM respectively. This latter value agrees well with

values previously reported (4, 7, 8). Precise determinations of the maximum velocity were difficult since substrate inhibition was frequently observed at the higher concentrations of OMP tested. This has previously been reported for OMP decarboxylase from calf thymus and was termed "substrate choking" (7). Figure 9 is the Lineweaver-Burk plot at pH 7.0 illustrating the existence of substrate inhibition for the yeast enzyme. The straight line shown was obtained by linear regression analysis that excluded the higher OMP concentration velocities that were subsequently added to the plot. Deviations from the expected linear Lineweaver-Burk plot are especially noticeable at OMP concentrations of 0.167 and 0.223 mM. Substrate inhibition was also evident at other pH values tested.

Figure 10 reveals how little the Michaelis constant for OMP and the maximum velocity for decarboxylation varied with pH. The experimental details for this study are given in the legend of the figure. The maximum velocity is apparently independent of pH over the region tested up to pH 7.5, after which it drops off rapidly. The K_m of OMP shows a very slight pH-dependence which may be negligible due to the magnitude of the error in the K_m.

Data presented in Chapter V suggested that OMP decarboxylase has more than one subunit. Hill plots (41) were constructed to see if any cooperativity existed between these subunits. Since no sigmoidicity was apparent in the presence of varying amounts of OMP (as seen by the linear double reciprocal plots), the Hill coefficient of 1.0 ± 0.1 for three separate determinations was not unexpected. The dissociation constant of OMP for these determinations was $5.3 \pm 0.1 \times 10^{-6}$ M.

Figure 9: Evidence of substrate inhibition of the OMP decarboxylase catalyzed reaction. 0.5 ug of phosphocellulose elutant was added to a 1 ml reaction mixture containing 0.011 umoles to 0.2230 umoles of OMP and the reaction followed spectrophotometrically as outlined under Methods. Results reported are the means and standard deviations for triplicate determinations at each OMP concentration.

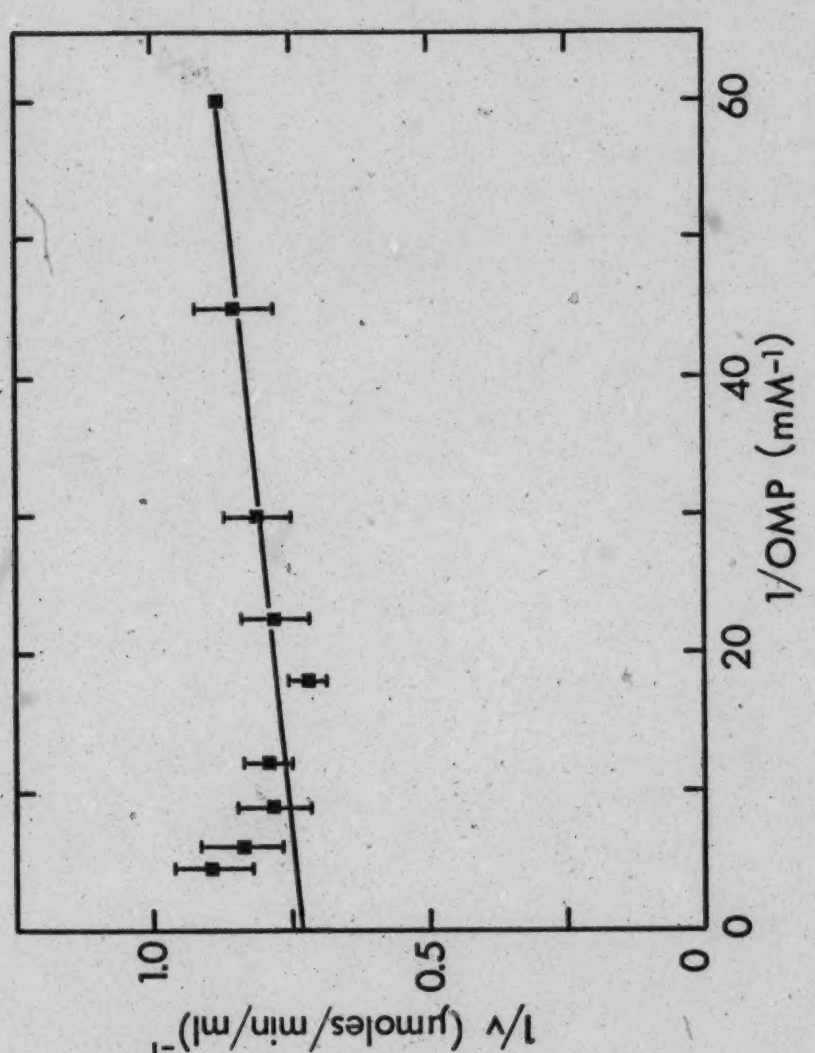
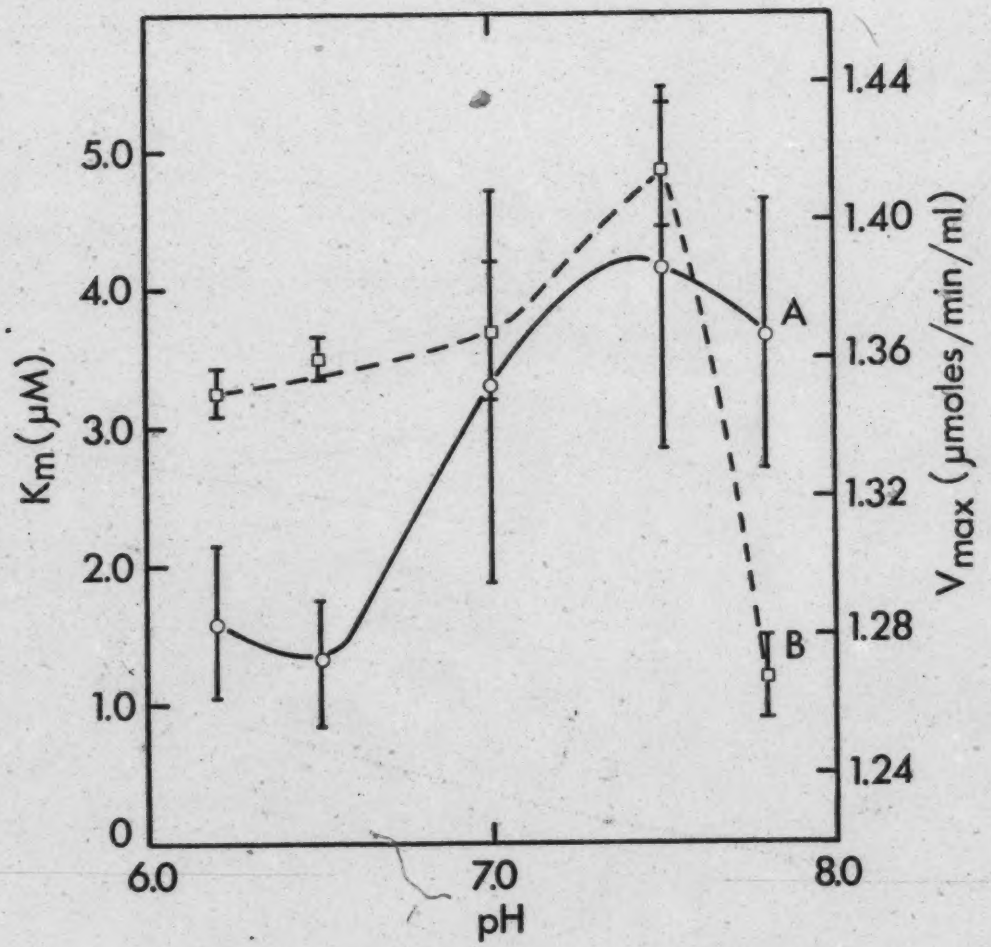

1/v (μmoles/min/ml)-1
1.0
0.5
0
0
20
40
60
1/OMP (mM-1)

Figure 10: Hydrogen ion effects on the Michaelis constant and maximum velocity of the reaction.

A -- K_m^{OMP}; B -- V_{max}. Assay media contained 0.5 ugm phosphocellulose elutant protein, 9-100 uM OMP in 85 mM KP - 5 mM EDTA at the selected pH. Spectrophotometric assays were run in triplicate at each OMP concentration used at each pH. V_{max} and K_m along with their standard errors were determined by the second program method outlined under Methods.


5.0
4.0
3.0
2.0
1.0
0
K_m (μM)
1.44
1.40
1.36
1.32
1.28
1.24
V_{max} (μmoles/min/ml)
A
B
6.0
7.0
8.0
pH

This value is very similar to the mean K_m value of OMP determined for this enzyme.

2. Reaction Reversibility

The millimolar extinction coefficient of OMP at pH 7.0 is 10.4 with the maximum absorbance occurring at 265 nm. For UMP at pH 7.0, a value of 10.0 at 262 nm is given in the Pabst circular (42). Analysis of the concentration of initial substrate and of the final product after the reaction had proceeded to completion indicated that there is essentially 100% conversion of OMP to UMP. Though this is an insensitive determination, it tends to support the previous conclusions of Lieberman et al. (2) and of Creasey and Handschumacher (4) that the decarboxylation of OMP is an irreversible reaction. Both groups examined the reversibility of the reaction by testing ^{14}C incorporation into OMP using isotopically labelled bicarbonate and UMP in the initial reaction medium. Since no radioactivity was detectable in OMP, they both separately concluded that the reaction was irreversible.

However, the rate at which OMP is decarboxylated leaves the conclusion of utter irreversibility open to question. Formation of low levels of OMP may not be readily detectable due to the rapid reversion to UMP and bicarbonate with respect to the rate of OMP formation. Measurements of isotope exchange into a pre-existing unlabelled pool of OMP provide a more sensitive test.

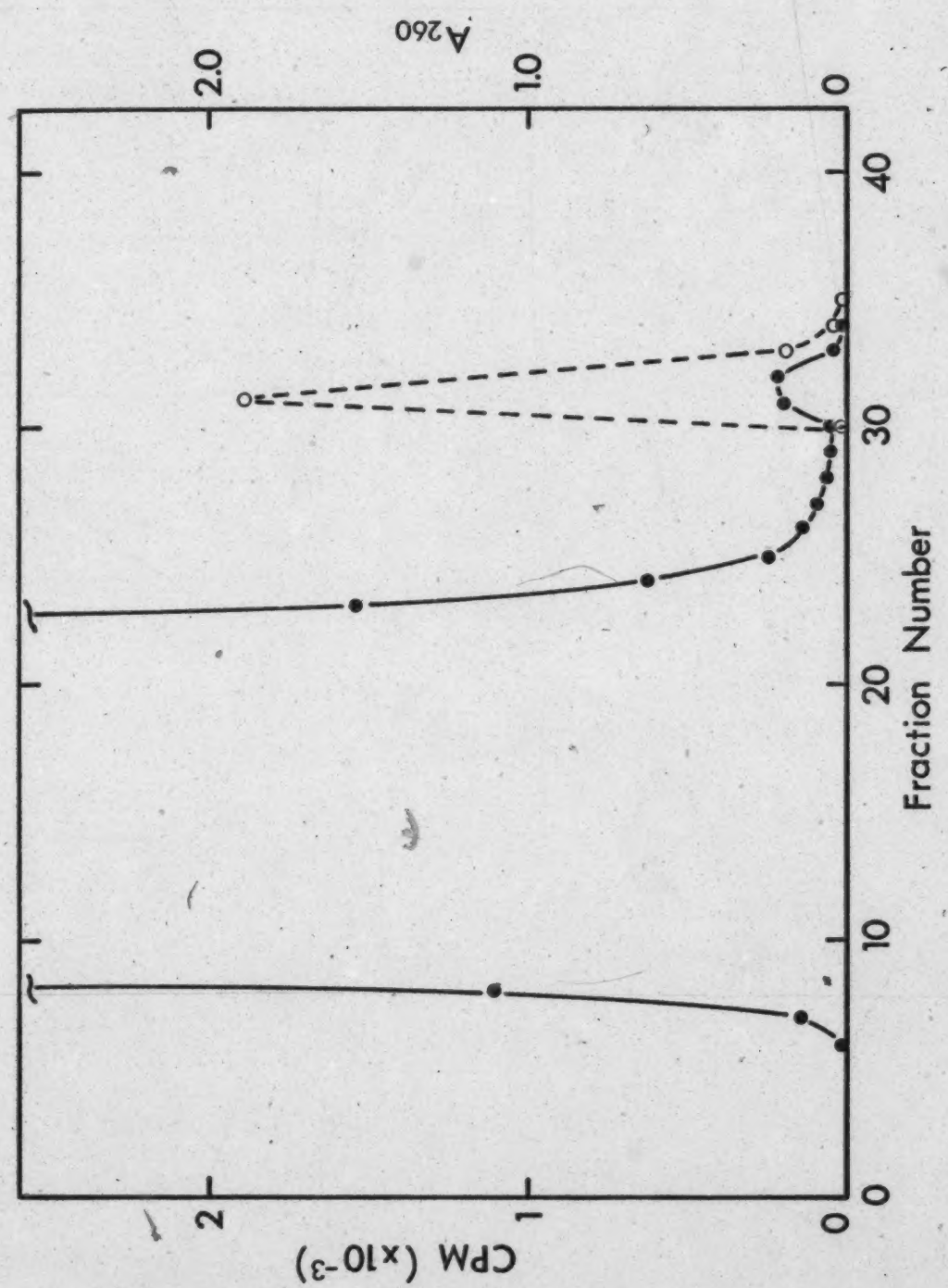
Accordingly, 1.74 umoles ^{14}C-$NaHCO_3$ (46 millicuries/millimole), 1.68 umoles UMP and 0.094 umoles of OMP (to serve as a trap for the possible labelled OMP formed) were mixed in 1050 ul of 85 mM KP - 5 mM EDTA, pH 7.5 to a total volume of 1.42 ml and the reaction was started by the addition of 1 ug protein. The mixture was incubated at 30°, 200 ul

aliquots were taken at various times and the reaction was stopped by the addition of 50 ul 2N KOH. The mixtures were subjected to chromatography on Dowex-1 columns (7.5 x 0.5 cm) with elution with 25 ml of 0.1M NH_4COOH followed by 15 ml of 1.0 M NH_4COOH. One milliliter fractions were collected. Absorbance at 260 nm was noted and radioactivity was determined. Figure 11 reveals the typical profile seen in each case. The height of the radioactive peak under that of the optical density peak was a measure of the ^{14}C incorporation that occurred. Since OMP and UMP elute together in this profile, the total optical density at 260 nm was essentially constant with each aliquot tested. The umoles of OMP remaining at the various times were calculated from a progress curve obtained by following the reaction spectrophotometrically at 285 nm under identical conditions using unlabelled bicarbonate. This provided a means of determining the specific radioactivity of the residual OMP.

The specific radioactivity of OMP was observed to increase with time. Fits to a linear increase of the specific radioactivity with time were poor as revealed by large variations in the value of the intercept by linear regression analysis. However, the slopes from these analyses from duplicate runs were equivalent to velocities of 0.39 and 0.65 umoles HCO_3^- being incorporated per hour per milliliter of protein. The apparent average velocity in the reverse direction is therefore approximately 6.6-12 nanomoles/min/ml protein with OMP serving as a ^{14}C-OMP trap. These results indicate that the decarboxylation of OMP is reversible to a certain degree, in agreement with the law of microscopic reversibility.

Under the experimental conditions used, the ratio of the velocity

Figure 11: Elution profile of ^{14}C-OMP from a Dowex-1 column by NH_4COOH. A 250 ul sample plus an equal volume of water was applied before the NH_4COOH elution was started. Experimental details are given in the text. The incubation time for this particular sample was 10 minutes. ^{14}C cpm. (—); A_{260}, (---).


A260
2.0
1.0
0
CPM (x10-3)
2
1
0
0
10
20
30
40
Fraction Number

in the forward direction (measured spectrophotometrically at 285 nm) to that of the reverse direction was 95. The magnitude of this ratio emphasizes the virtual irreversibility of the OMP decarboxylation reaction. Thus the improbability of detection of OMP formation from bicarbonate and UMP in the absence of an OMP trap as previously attempted is apparent.

OMP decarboxylase is a highly specific enzyme known to decarboxylate only orotidylate and 5-azaorotidylate (10). The substrate analogs, orotidine and orotic acid, were tested for their ability to inhibit decarboxylation. Fifty per cent inhibition was seen with 0.8 mM orotidine by interpolation of a plot of percentage inhibition versus the orotidine concentration at a fixed OMP concentration. Orotic acid (0.24 mM) gave only 6.5% inhibition but higher concentrations could not be used due to both the limited solubility and the high ultraviolet absorbance of orotic acid.

Orotidine is believed to be metabolically inert (43). This was partially confirmed by the observation that incubation of 1.6 mM orotidine with OMP decarboxylase resulted in no spectrophotometric change at 285 nm over a one-hour period, showing that the nucleoside is not decarboxylated to uridine. The presence of the phosphate at the 5'-position of the ribofuranose ring thus appears essential for the binding of the nucleotide in the proper orientation (that enables decarboxylation to proceed). The large ratio of the orotidine concentration giving 50% inhibition (0.8 mM) to the dissociation constant of orotidylic acid (5.3 uM) reflects the magnitude of the binding enhancement that the phosphate moiety provides. It is noteworthy that the most effective inhibitors

of OMP decarboxylase are primarily 5' nucleoside monophosphates (6, 8).

The near irreversibility of the reaction severely limits the kinetic methods applicable to the study of this enzyme. It is impossible to do initial velocity studies in the direction of OMP synthesis. The use of isotope exchange studies at chemical equilibrium is likewise impractical. The only useful kinetic tool remaining for elucidating reaction sequence possibilities is that of product inhibition. In studying the effects of UMP and HCO_3^-, potassium chloride was used to maintain an effectively constant ionic strength. Studies with UMP (as a product inhibitor) were carried out at pH 7.0 or 7.5 while a pH of 7.5 was used to help minimize conversion of the HCO_3^- to CO_2 gas or carbonate.

3. UMP Inhibition Studies

Although kinetic analysis of the product inhibition by UMP had not been determined, several authors have reported data suggesting that UMP is a competitive inhibitor of OMP binding (4, 6, 40). The latter paper reported a K_i of 2.08×10^{-3} M with UMP for the yeast enzyme. Statistical methods were not used to verify that the inhibition was indeed competitive.

Replicate measurements of the reaction velocity in the presence of varying concentrations of UMP indicated that UMP was not a competitive inhibitor, as shown by the data of Figure 12. The presence of UMP causes a change in the intercept of the reciprocal plot which far exceeds the standard error of the maximum velocity. Thus it would appear that the binding of UMP is not exclusively restricted to the free enzyme.

Strikingly, the intercept plot as a function of the UMP concentration was not linear (as seen in Figure 13). This figure implies that

Figure 12: Double reciprocal plots showing product inhibition by UMP at various OMP concentrations. Results are reported as the mean, plus or minus the standard deviation obtained for three velocity determinations at each selected OMP and UMP concentrations. A, 0.0 mM UMP; B, 0.75 mM UMP; C, 1.29 mM UMP; D, 2.64 mM UMP; E, 3.20 mM UMP.

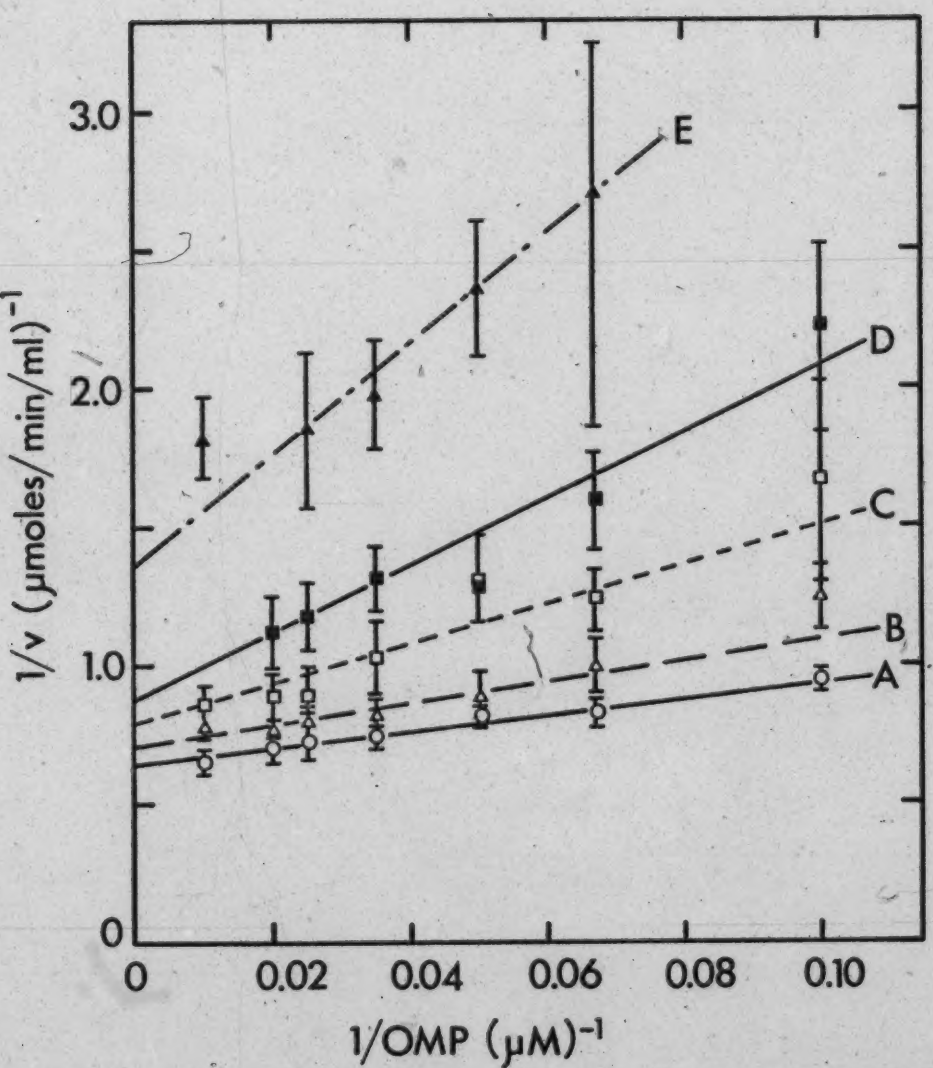

3.0
2.0
1.0
0
0
0.02
0.04
0.06
0.08
0.10
1/v (μmoles/min/ml)⁻¹
1/OMP (μM)⁻¹
E
D
C
B
A

Figure 13: Plot of the intercepts from Figure 12 obtained from a Wilkinson weighted fit to the Lineweaver-Burk equation against the UMP concentration. Deviations given are based on the standard error reported in the V_{max} determination.

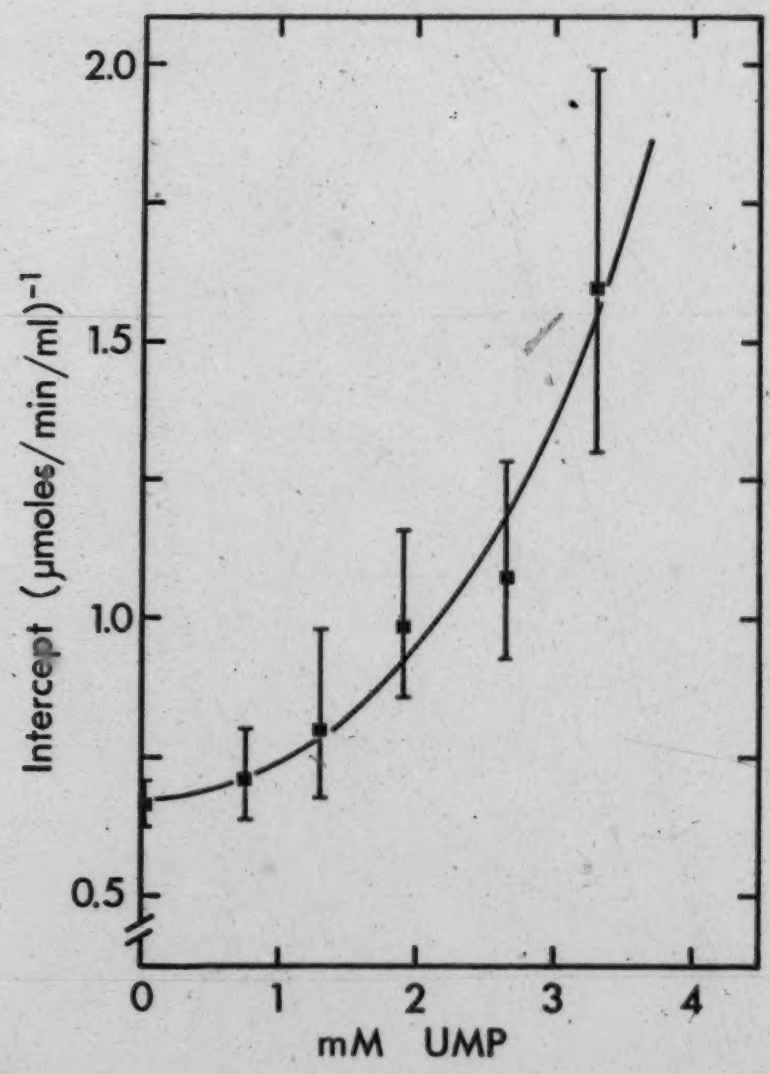
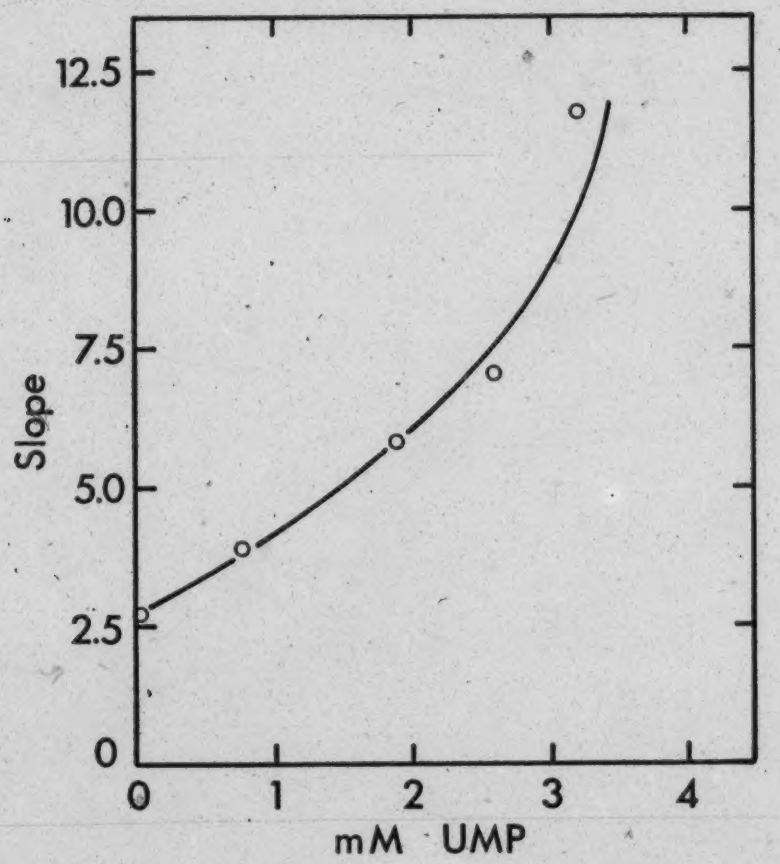

2.0
1.5
1.0
0.5
Intercept (μmoles/min/ml)$^{-1}$
0
1
2
3
4
mM UMP

Figure 14: Plot of the slopes obtained from Figure 12 against the UMP concentration. Slopes were equated to K_m/V_{max} obtained by the weighted fit program (Method 2). Standard errors in the slope determination are not reported since neither program used provides an accurate means of assessing them. A standard error for the slope could be used from the Wilkinson fit if the % error in the V_{max} and K_m determinations were summed before the K_m was divided by V_{max}. This method gave an average error of 15 - 20% in the value of the slope obtained.


12.5
10.0
7.5
5.0
2.5
0
Slope
0
1
2
3
4
mM UMP

either a dead end inhibitor complex is formed with UMP or that some other degree of complexity in the reaction sequence exists that cannot be explained in terms of a simple ordered Uni-Bi mechanism.

Analysis of the slope replot, shown in Figure 14, gave an inhibitory constant (K_i) of 1.6 mM.

4. HCO_3^- Inhibition Studies

The double reciprocal plots of the rate of OMP decarboxylation at various concentrations of HCO_3^- are shown in Figure 15. Figure 16 gives the slope and intercept replots obtained from this figure. The inhibition by bicarbonate appears to be competitive with no statistically significant changes in the intercept value. However, the intercept value at 300 mM bicarbonate was significantly different from those of the lower bicarbonate concentrations. Extrapolation of the slope replot gives an inhibition constant of approximately 375 mM. The apparent weakness of the inhibitory effect of bicarbonate, as evidenced by the high concentrations required, may obscure the precise nature of the inhibition by this product.

5. Inhibition Studies With Both Products Present

Bicarbonate, which appears to be a competitive inhibitor in the absence of UMP (as seen from the intercept replots in Figures 16 and 17A) apparently becomes a mixed competitive-non-competitive inhibitor in the presence of UMP. This transition is likely a consequence of the non-competitive inhibitory nature of UMP.

Slope replots also appear to be linear functions of UMP or HCO_3^- when used in combination as shown in Figure 17B. A slight tendency of the slope replot toward non-linearity appears evident as the concentration of the fixed product is increased.

Figure 15: Double reciprocal plots showing product inhibition by bicarbonate at varying OMP concentrations. Results are plotted as the means plus or minus the standard deviation obtained from three or more determinations at each selected initial concentration.
A, 0 mM $KHCO_3$; B, 150 mM $KHCO_3$; C, 225 mM $KHCO_3$; D, 300 mM $KHCO_3$. KCl solutions of 300, 150, 75 and 0 mM were added respectively to keep the ionic strength constant.

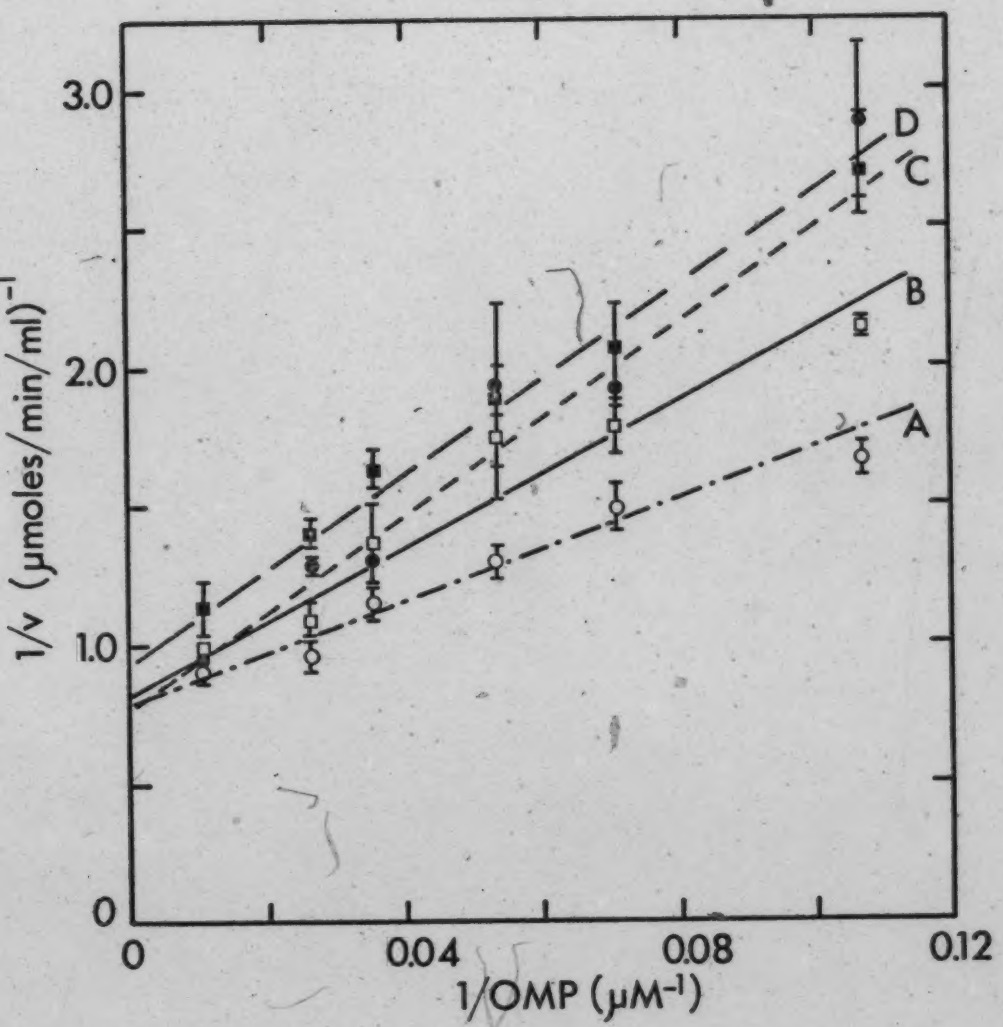
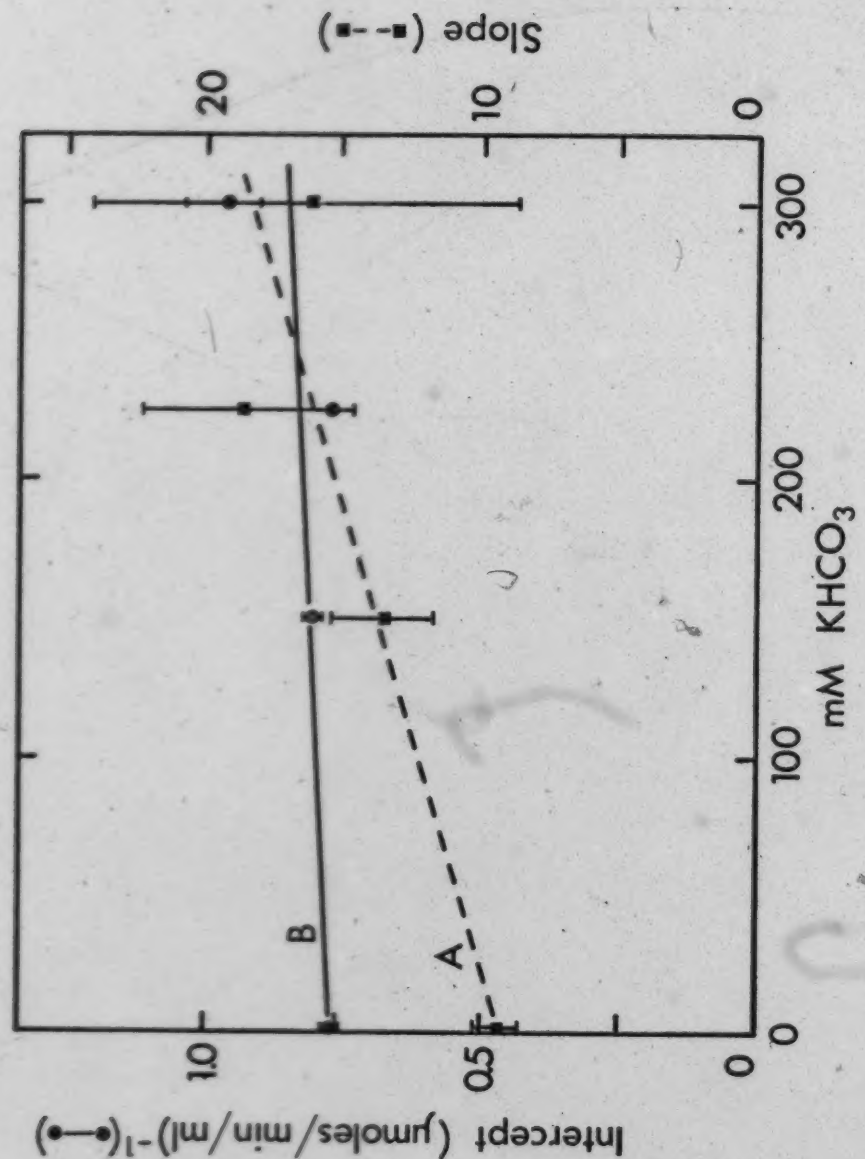

3.0
2.0
1.0
0
1/v (μmoles/min/ml)⁻¹
0
0.04
0.08
0.12
1/OMP (μM⁻¹)
D
C
B
A

Figure 16: Plots of the slopes (A) and intercepts (B) obtained from Figure 15 against the bicarbonate concentration. Standard errors are from the Wilkinson weighted fit program. The error in the slope was expressed as a percentage equal to the sum of the percentage errors in the V_{max} and K_m respectively.


Slope (■--■)
20
10
0
300
200
100
0
mM $KHCO_3$
B
A
1.0
0.5
0
Intercept (μmoles/min/ml)$^{-1}$(●—●)

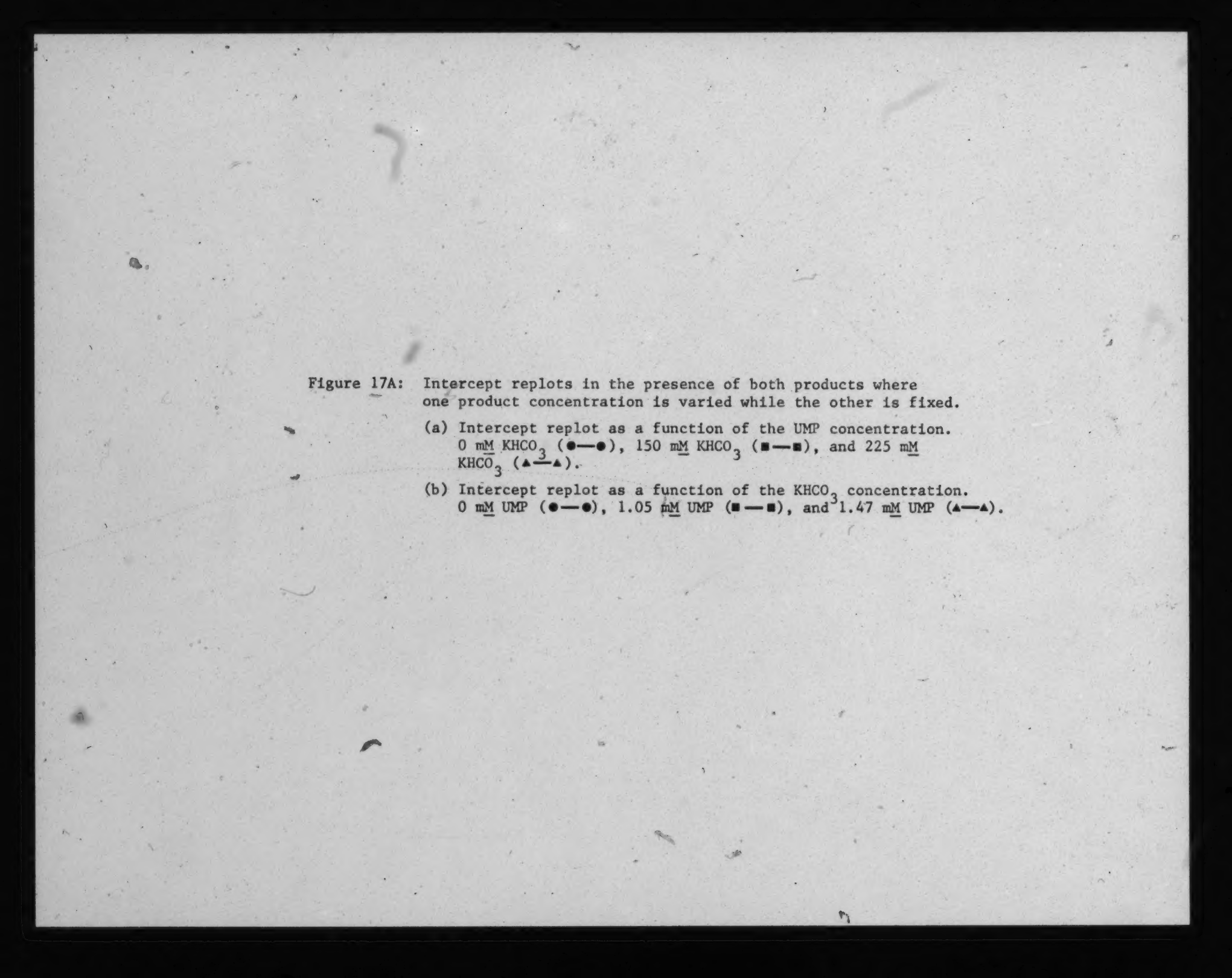


Figure 17A: Intercept replots in the presence of both products where one product concentration is varied while the other is fixed.

(a) Intercept replot as a function of the UMP concentration. 0 mM $KHCO_3$ (●—●), 150 mM $KHCO_3$ (■—■), and 225 mM $KHCO_3$ (▲—▲).

(b) Intercept replot as a function of the $KHCO_3$ concentration. 0 mM UMP (●—●), 1.05 mM UMP (■—■), and 1.47 mM UMP (▲—▲).

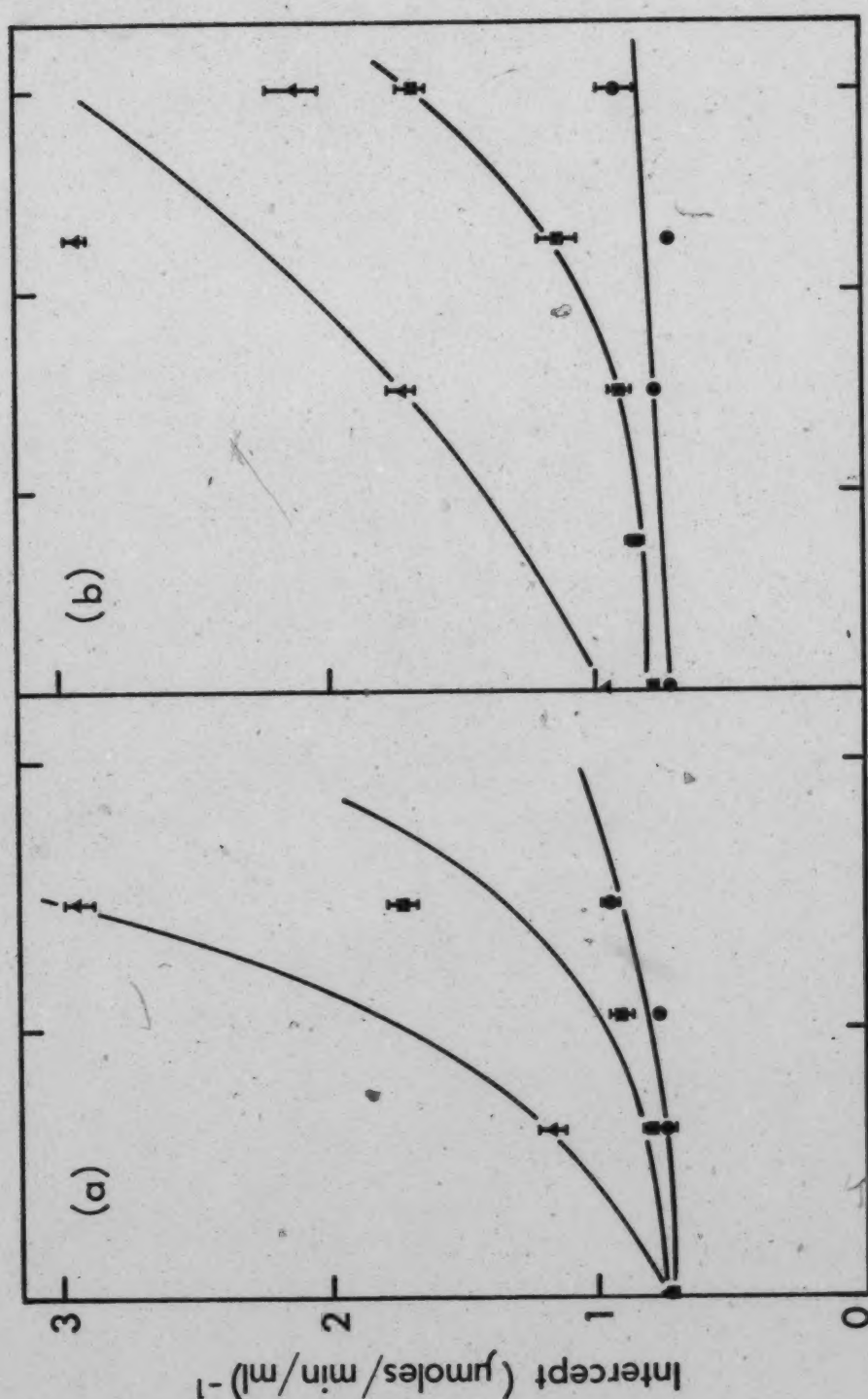
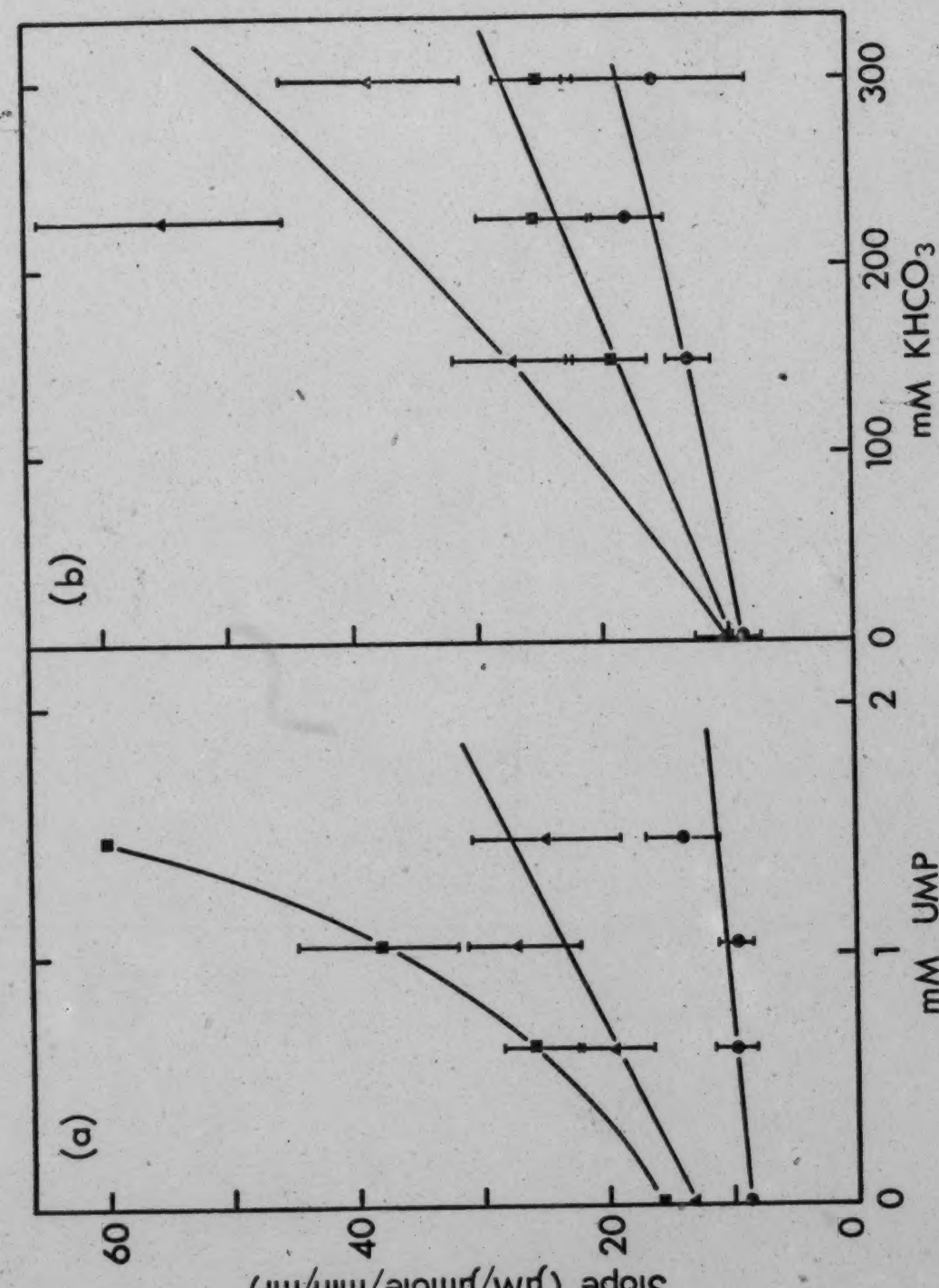

Intercept (μmoles/min/ml)-1
3
2
1
0
(a)
0
1
2
mM UMP
(b)
0
100
200
300
mM KHCO3

Figure 17B: Slope replots in the presence of both products where one product concentration is varied while the other is fixed.

(a) Slope replots as a function of the UMP concentration. 0 mM $KHCO_3$ (●—●), 150 mM $KHCO_3$ (▲—▲), and 300 mM $KHCO_3$ (■—■).

(b) Slope replots as a function of the $KHCO_3$ concentration. 0 mM UMP (●—●), 0.62 mM UMP (■—■), 1.05 mM UMP (▲—▲).


Slope (μM/μmole/min/ml)
60
40
20
0
(a)
0
1
2
mM UMP
(b)
0
100
200
300
mM KHCO3

The possibility of co-operativity between enzyme subunits contributing to the non-linear plots appears to be slim since the Hill interaction coefficient was 1.0 $\pm$ 0.1 with the ratio of the Michaelis constant of OMP to the dissociation constant of OMP equalling 1.0 $\pm$ 0.04, as shown in Tables III and IV, regardless of the inhibitor concentrations used.

6. Mechanism Proposal

Upon application of Cleland's rules and nomenclature (36, 37, 38) to the study of the reaction sequence, the following conclusions were deduced. The reaction could not be simply an ordered Uni-Bi reaction mechanism since one product should give linear competitive inhibition while the other would give linear non-competitive inhibition. In an "Iso Uni-Bi" reaction mechanism (with isomerization between two enzyme forms after the release of both products) both products would show linear non-competitive inhibition patterns not observed here. If the mechanism involved the rapid random release of products, after the slow conversion of the substrate to products, both products should display linear competitive inhibition patterns not seen here.

The existence of a dead end inhibition complex or complexes involving UMP has already been suggested. Since bicarbonate appears to be a competitive product inhibitor (in the absence of UMP as seen in Figures 15 and 17A), two possible dead end inhibitor complexes with UMP are shown in Figure 18.

TABLE III

Hill Coefficient Determined Under Various Inhibitory Conditions

mM KHCO$_3$ \ mM UMP	0	0.62	1.04	1.47
0	0.989	0.970	0.984	1.210
74.7	1.094	1.003	N.D.	N.D.
149	0.980	N.D.	1.020	N.D.
224	1.041	N.D.	N.D.	N.D.
299	1.042	N.D.	1.053	0.985

N.D. – not determined.

TABLE IV

Ratio of Km^{OMP}/K_d^{OMP} Determined Under Various Inhibitory Conditions

mM $KHCO_3$ \ mM UMP	0	0.62	1.04	1.47
0	1.096	1.051	1.033	1.115
74.7	1.023	0.943	N.D.	N.D.
149	1.006	N.D.	1.016	N.D.
224	1.080	N.D.	N.D.	N.D.
299	1.000	N.D.	1.000	1.049

N.D. - not determined.

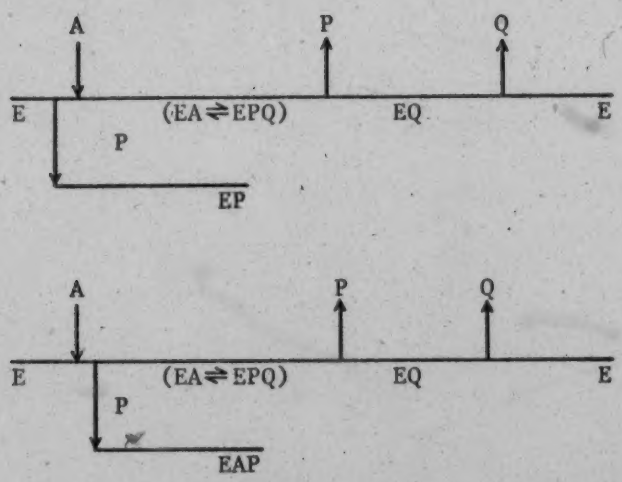


Figure 18. Schematic representation of two possible dead end inhibitor complexes with a product inhibitor where A, P, and Q represent OMP; UMP and HCO_3^- respectively.

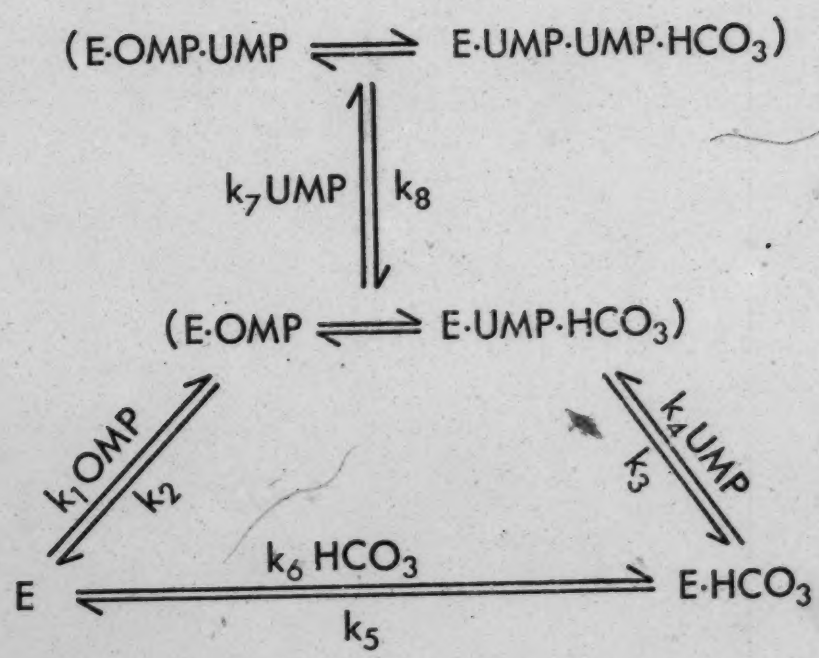
In the absence of bicarbonate, representation I of Figure 18 would display slope-parabolic-intercept-linear non-competitive inhibition with UMP as the product inhibitor, while representation II would give slope-linear-intercept-parabloic non-competitive inhibition.

Thus dead end inhibitor complex as shown in representation II of Figure 18 appears consistent with the product inhibition patterns seen. The full rate equation (equation I) derived by the King Altman method (44) gives the kinetic expression for the reaction sequence as shown in Figure 19 in terms of the rate constants for the individual steps of the sequence.

$$v = \frac{(k_1k_3k_5A - k_2k_4k_6PQ)E_t}{k_5(k_2+k_3) + k_1(k_3+k_5)A + k_6(k_2+k_3)Q + k_2k_4P + k_1k_4\left(\frac{1+k_7P}{k_8}\right)AP} \quad (I)$$

$$+ k_4k_6\left(\frac{1+k_7P}{k_8}\right)PQ + \frac{k_1k_5k_7AP}{k_8}$$

Figure 19: Schematic representation of a probable reaction mechanism of OMP decarboxylase involving a dead end inhibitor complex with the central complex. Rate constants in the forward direction are k_1, k_2, k_5, and k_7; and in the reverse k_2, k_4, k_6, and k_8.


(E·OMP·UMP ⇌ E·UMP·UMP·HCO3)
k7UMP
k8
(E·OMP ⇌ E·UMP·HCO3)
k1OMP
k2
k4UMP
k3
E
k6 HCO3
k5
E·HCO3

Equation I can be transformed to

$$v = \frac{V_1V_2\left(A-\frac{PQ}{K_{eq}}\right)}{K_aV_2 + V_2A + \frac{K_pV_1Q}{K_{eq}} + \frac{K_qV_1P}{K_{eq}} + \frac{V_2AP}{K_{ip}} + \frac{V_1PQ}{K_{eq}} + \frac{V_2AP^2}{K_iK_{ip}}2 + \frac{V_1P^2Q}{K_{eq}K_{ip}}3} \quad (II)$$

where the constants are defined in terms of coefficients as outlined by Cleland (36) with the unusual coefficient terms $K_{ip}^{\ 2}$ and $K_{ip}^{\ 3}$ representing the coefficient of AP over the coefficient of AP^2 and the coefficient of PQ over the coefficient of P^2Q respectively. Equation II, rewritten in reciprocal form in the forward direction only, is:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{Q}{K_{iq}} + \frac{K_qP}{K_pK_{iq}} + \frac{PQ}{K_pK_{iq}} + \frac{P^2Q}{K_pK_{iq}K_{ip}}3\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{P}{K_{ip}} + \frac{K_qP^2}{K_{ip}K_{ip}}2\right) \quad (III)$$

In the absence of the products P and Q, equation III reduces to:

$$\frac{1}{v} = \frac{K}{V_1A} + \frac{1}{V_1} \quad (IV)$$

In this form the maximum velocity in the forward direction the Michaelis constant for OMP can be determined directly. In the absence of the product P, equation III reduces to:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{Q}{K_{iq}}\right)\frac{1}{A} + \frac{1}{V_1} \quad (V)$$

Thus bicarbonate (Q) should be a simple competitive inhibitor in the absence of UMP in agreement with the results seen in Figures 15 and 17A.

In the absence of the product Q, equation III reduces to:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_qP}{K_pK_{iq}}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{P}{K_{ip}} + \frac{P^2}{K_{ip}K_{ip}}2\right) \quad (VI)$$

Thus a slope-linear intercept-parabolic non-competitive inhibition should

be seen with the product P present. This is in agreement with the results observed with UMP in Figures 13, 14, 17A and 17B.

From equation III, with both products present, it can be seen that both slope and intercept plots are parabolic functions of the product P. With the product Q, the intercept, which ordinarily depends on the concentration of P and should be unchanged by Q, may be affected by the ability of Q to keep the enzyme in the intermediate complexes to which P binds thereby reducing the effective free enzyme concentration.

From equation III, with both products present, it can be seen that both slope and intercept are parabolic functions of the product P. With the product Q, the intercept should not be affected while the slope should be a linear function of Q. The results observed in Figures 17A and 17B are not completely consistent with these predictions. However, the intercept, which ordinarily depends on the concentration of P and should be unchanged by Q, may be affected by the ability of P to keep the enzyme in the intermediate complexes thereby reducing the effective free enzyme concentration. The high K_{iq} value of 380 mM determined in Figure 16 may obscure any curvature in the slope plots until higher concentrations of the product Q are reached. Similarly the slope replot, which should be linear function of Q, may be affected by high concentrations of P thereby giving it a non-linear form due to the P^2Q term. The results observed in Figures 17A and 17B are consistent with this.

Thus, it appears that the results obtained for the OMP decarboxylase catalyzed reaction can be explained in terms of an ordered Uni-Bi reaction sequence where UMP and then bicarbonate are released from the

enzyme with UMP being capable of forming a dead end complex with the central complex. Table V gives the kinetic constants that could be determined for this reaction sequence. Reasons for the irreversibility of the OMP decarboxylation are not apparent from the constants shown.

The results indicate the necessity for precise data in the kinetic treatment of enzymic reactions. Authors concluding that UMP was a competitive inhibitor of OMP (4, 39) could have been working within the UMP concentration range where the determined maximum velocity does not appear to differ significantly from the maximum velocity seen in the absence of UMP. Maximum velocities must be shown to be significantly different before the inhibition can be termed "non-competitive." At higher UMP concentrations, Figure 12 reveals that UMP cannot be termed a competitive inhibitor.

This study has revealed the difficulties involved in elucidating a plausible reaction sequence when the tools available for the study are limited by the peculiar properties of that system.

TABLE V

Kinetic Constants Obtained for OMP Decarboxylase

K_A	5.1×10^{-6} M
K_d^A	5.3×10^{-6} M
V_1	1.4×10^{-6} Moles/min/ml
K_{iq}	0.38 M
$\frac{K_q}{K_p K_{iq}}$	1.6×10^{-3} M

A, Q, and P refer to OMP, HCO_3^- and UMP respectively.

VII. SULFHYDRYL GROUPS OF OMP DECARBOXYLASE

A. Introduction

OMP decarboxylase from yeast differs from the enzyme from other sources in its sensitivity to sulfhydryl reagents. The Neurospora crassa (12), and rat liver (4) enzymes do not display great sensitivity to either N-ethylmaleimide (NEM) or p-chloromercuribenzoate (p-CMB). Creasey and Handschumacher (4) have previously reported on the sensitivity of the yeast enzyme to the sulfhydryl reagents p-CMB and NEM. A more detailed study of sensitivity of yeast OMP decarboxylase to sulfhydryls was undertaken.

B. Methods

The initial studies involved adding a fixed concentration of either NEM or iodoacetamide (IAA) to a 1 ml cuvette containing enzyme, in 85 mM KP - 1 mM EDTA, pH 7.00 and starting the reaction by adding a saturating concentration of OMP. The reaction was followed in a Gilford 240 spectrophotometer at 285 nm.

For inactivation studies, 90 ug of protein eluted from phosphocellulose were incubated at 30° in 85 mM KP-5 mM EDTA, pH 7.0 in the presence of NEM or IAA in a total volume of 1.5 ml. With IAA, the protein was pre-incubated for 15-20 minutes at 30° before the sulfhydryl reagent was added. A control mixture contained 60 ug of protein in 1 ml of the same buffer. Activity of these mixtures was determined by placing 100 ul samples into 740 ul of 85 mM KP - 1 mM EDTA, pH 7.0 containing 26.5 nmoles of OMP and following the reaction spectrophotometrically at 285 nm, using the millimolar extinction coefficient change of -1.93. Samples

were taken before the addition of sulfhydryl reagent to determine the initial activity (100% value) of the enzyme. For subsequent samples, the ten-fold dilution of inhibitor upon addition to the assay mixture reduced further inhibitiory effects to negligible levels.

Pseudo-first-order rate constants for inactivation were obtained from plots of the logarithm of the residual activity versus time (45). Second order rate constants were obtained by dividing the pseudo-first-order rate constant by the concentration of the sulfhydryl reagent.

C. Results

OMP decarboxylase displayed equal sensitivity to both iodoacetamide and N-ethylmaleimide, being inactivated 80-90% with 0.1 mM concentrations of each after a 10 minute exposure. The addition of a twenty-fold molar excess of 2-mercaptoethanol with respect to that of the sulfhydryl reagent used prevented the reagent from inactivating the enzyme, although 2-mercaptoethanol alone did not increase enzymatic activity.

1. Sulfhydryl Studies with N-ethylmaleimide

The addition of NEM in a two-fold molar excess to 0.2 mM OMP did not inactivate the enzyme (6ug/ml) if the catalytic reaction had been started before the NEM addition. However, the addition of NEM before the addition of an equal amount of OMP resulted in a progressive inactivation of the enzyme, the degree of inactivation being dependent on the time elapsed between the NEM and OMP additions. Thus the sulfhydryl reagent NEM cannot be used to stop a proceeding reaction.

N-ethylmaleimide concentrations of 0.29, 0.44, and 0.73 millimolar resulted in greater than 90% loss of activity within 25 minutes. This high sensitivity to these reagents reveals that concentrations

lower than those normally involved in sulfhydryl inactivation studies must be used.

With 0.033 mM NEM, OMP decarboxylase inactivation follows a pattern that is biphasic in nature as shown in Figure 20.

210 uM OMP, 10.1 mM UMP, 8.8 mM orotidine or 0.32 mM orotic acid did not offer any protection to the enzyme from NEM activation. Since the OMP is rapidly converted to UMP in the incubation medium, its possible protective effect is quickly minimized by the decarboxylation that proceeds. Higher concentrations of orotic acid were not attainable because of its limited solubility. The ineffectiveness of orotidine and UMP in providing protection from NEM inactivation suggests that the sensitive sulfhydryl group is not part of the enzyme's active site. Inactivation could be a result of a conformational change invoked by the binding of NEM to the protein.

Three separate determinations of the pseudo-first-order rate constant for the fast phase of inactivation gave values of 0.157, 0.215 and 0.203 min^{-1} respectively. The average value of the second-order rate constant of inactivation was $5.8 \pm 0.9 \times 10^3$ $min^{-1}M^{-1}$ indicating that the proposed sulfhydryl group reacts very quickly with NEM.

2. Sulfhydryl Studies with Iodoacetamide

High reactivity of a sulfhydryl group with iodoacetamide may be taken as evidence of its availability to the solvent (46). The rate of reaction with iodoacetamide of glutathione in solution was 75 $min^{-1}M^{-1}$. Comparable rates of reaction of enzymatic sulfhydryl groups is indicative of the unhindered accessibility on the surface of the molecule (46, 47). The rapid reactivity of OMP decarboxylase with NEM suggested that measurements of the rate of IAA reaction with OMP decarboxylase might

Figure 20: OMP decarboxylase sulfhydryl lability at 30° C in the presence of 0.033 mM NEM. 100 ul aliquots from the incubation mixtures outlined were added to 700 ul of 85 mM KP - 1 mM EDTA, pH 7.0 and 20 ul of 1.327 mM OMP. The reaction was followed as outlined under Methods.

	Control	Sample
85 mM KP - 5 mM EDTA, pH 7.0	600 ul	1100 ul
85 mM KP - 1 mM EDTA, pH 7.0	300 ul	235 ul
3.33 mM NEM, pH 7.0	--	15 ul
620 ug/ml phosphocellulose protein	100 ul	150 ul
Total volume	1000 ul	1500 ul

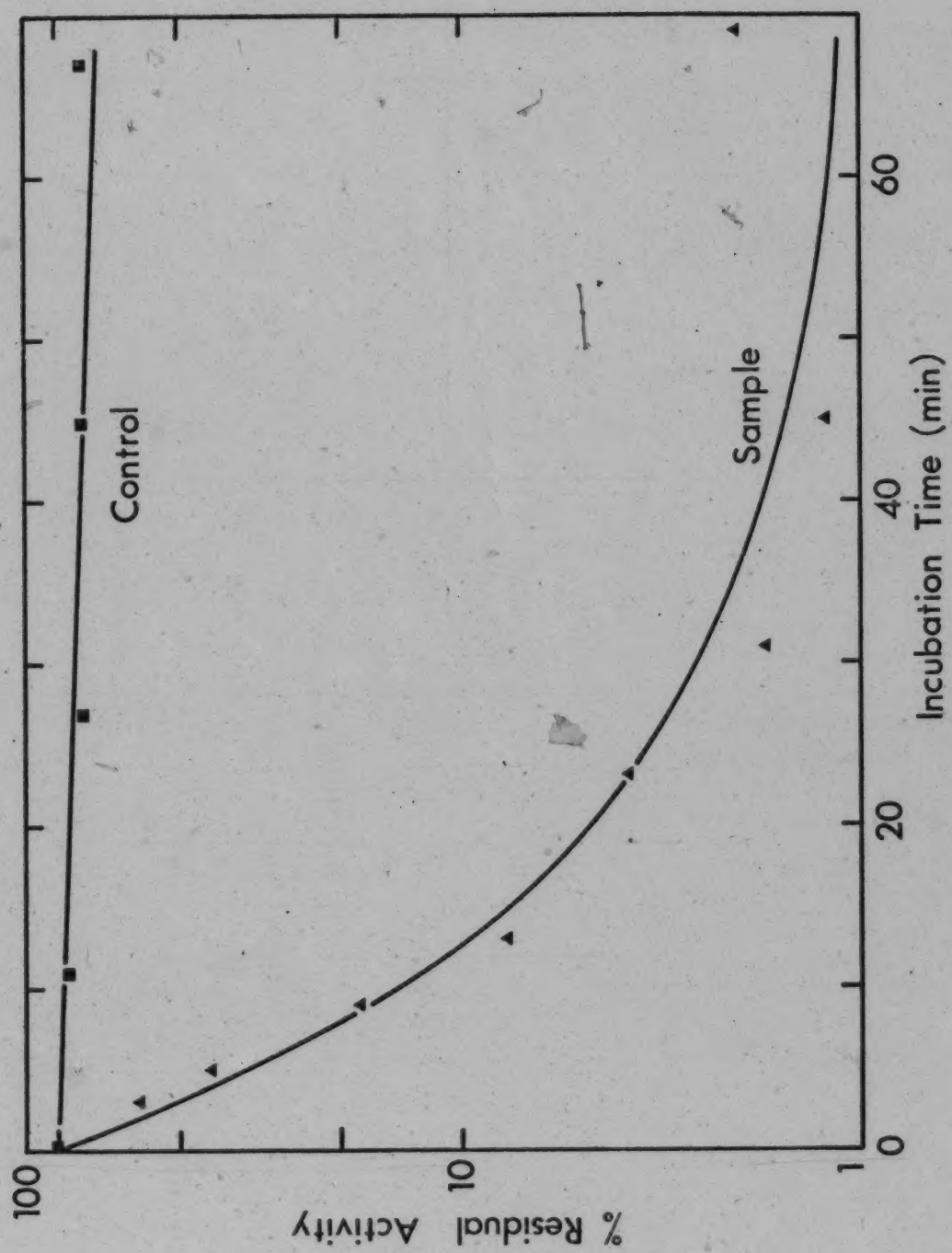

% Residual Activity
100
10
1
0
20
40
60
Incubation Time (min)
Control
Sample

Figure 21: Progressive inactivation of OMP decarboxylase by iodoacetamide, a specific sulfhydryl complexing reagent. The experimental design was the same as that of Figure 21 with IAA replacing NEM. A - Control with no IAA. B - with 0.47 mM IAA.

% Residual Activity
100
10
1
A
B
0
100
200
300
Incubation Time (min)

reveal the availability of these SH groups to the aqueous media. Again the biphasic nature of the OMP decarboxylase inactivation was observed, as shown in Figure 21. Evaluation of the rate constants for three different experimental runs with IAA are shown in Table VI. The mean values of the second-order rate constants for these experiments were 124 ± 9 $\underline{M}^{-1}min^{-1}$ and 21.9 ± 4.0 $\underline{M}^{-1}min^{-1}$ for the fast and slow reacting sulfhydryls respectively. The magnitude of these two values indicates that neither of these SH groups is buried within the protein.

A fourth experiment was run simultaneously with the first. The presence of orotidine in this incubation system has no appreciable effect on the reaction rates of either group with iodoacetamide.

Ray and Koshland (48) have previously shown four possible inhibition patterns are theoretically possible, depending on how the modification of amino acid residues affects enzymatic activity. The method is particularly applicable when only one type of residue is being specifically modified by a reagent. The biphasic inhibition pattern of Figure 21 for OMP decarboxylase follows that proposed for case 4 by these authors. In this inhibition scheme, illustrated in Figure 22, the reaction of one residue with the modifying reagent still gives a protein that has partial retention of enzymatic activity. Further modification of the enzyme by reagent results in the complete loss of activity.

It is noteworthy that this figure is symmetrical. The pathway that predominates will depend on the magnitude of k_1 and k_2. The complexity of the kinetics of inactivation will therefore largely depend on the fractional activity left in the modified forms E_1 and E_2.

TABLE VI

Rate Constants for the Inactivation of OMP Decarboxylase by IAA

Experiment number	Iodoacetamide incubation concentration (mM)	Pseudo k_1 of fast SH (min^{-1})	Pseudo k_1 of slow SH (min^{-1})	k_2 of fast SH ($min^{-1}M^{-1}$)	k_2 of slow SH ($min^{-1}M^{-1}$)
1	0.497	0.0578	0.0122	116	24.6
2	0.474	0.0642	0.0113	135	23.8
3	0.474	0.0570	0.0082	120	17.3
4[a]	0.497	0.0536	0.0101	108	20.2

[a] This incubation was carried out in the presence of 4.72 mM orotidine.

Experimental detail is outlined under methods.

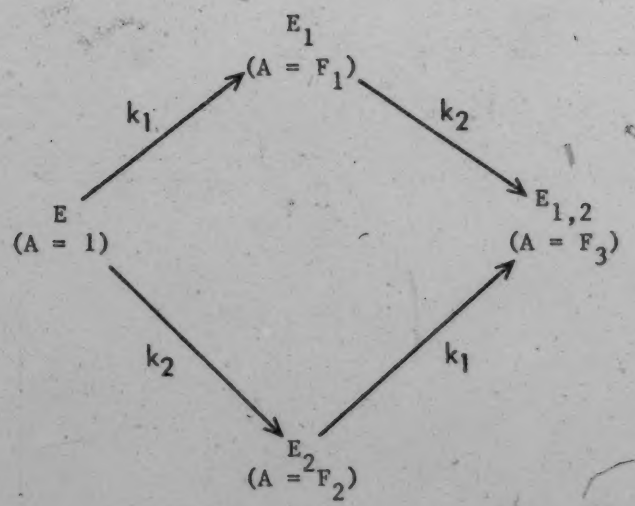


Figure 22. Mechanism for partial retention of enzyme activity upon reagent modification of specific residues. F_1,F_2, and F_3 refer to fractions of enzymatic activity left to the protein as a result of the modification of a given residue. The activity of the unmodified protein was arbitrarily given a fractional level of 1.00. k_1 and k_2 are pseudo-first-order inactivation rate constants.

The observed activity as a function of time for this case is given by Equation I.

$$A/A_0 = F_3 + (1-F_1-F_2+F_3)e^{-(k_1+k_2)t} + (F_1-F_3)e^{-k_2t} + (F_2-F_3)e^{-k_1t} \quad (I)$$

Precise evaluation of the constants is feasible only when $F_2 = F_3 = 0$ or $F_1 = F_3 = 0$. Figure 23 indicates the method for graphical analysis for this case as applied to the mean of the fractional activity left at various times for experiments one to three of Table VI assuming $F_2 = F_3 = 0$. Table VII gives the values of Table VI reassessed in accordance to Equation I.

From the results tabulated in Table VII, the generalized Equation I for case 4 in the case of OMP decarboxylase is simplified to Equation II.

$$A/Ao = 0.911e^{-0.0597t} + 0.089e^{-0.0106t} \quad (II)$$

Figure 23: Kinetics of the loss of catalytic activity of OMP decarboxylase by SH modification according to Equation I.

(●) represents the mean and standard deviation of the fractional activity left at various times from experiments I to III from Table VI and (○—○) the values calculated at selected times according to Equation II. The line drawn is from these latter values.

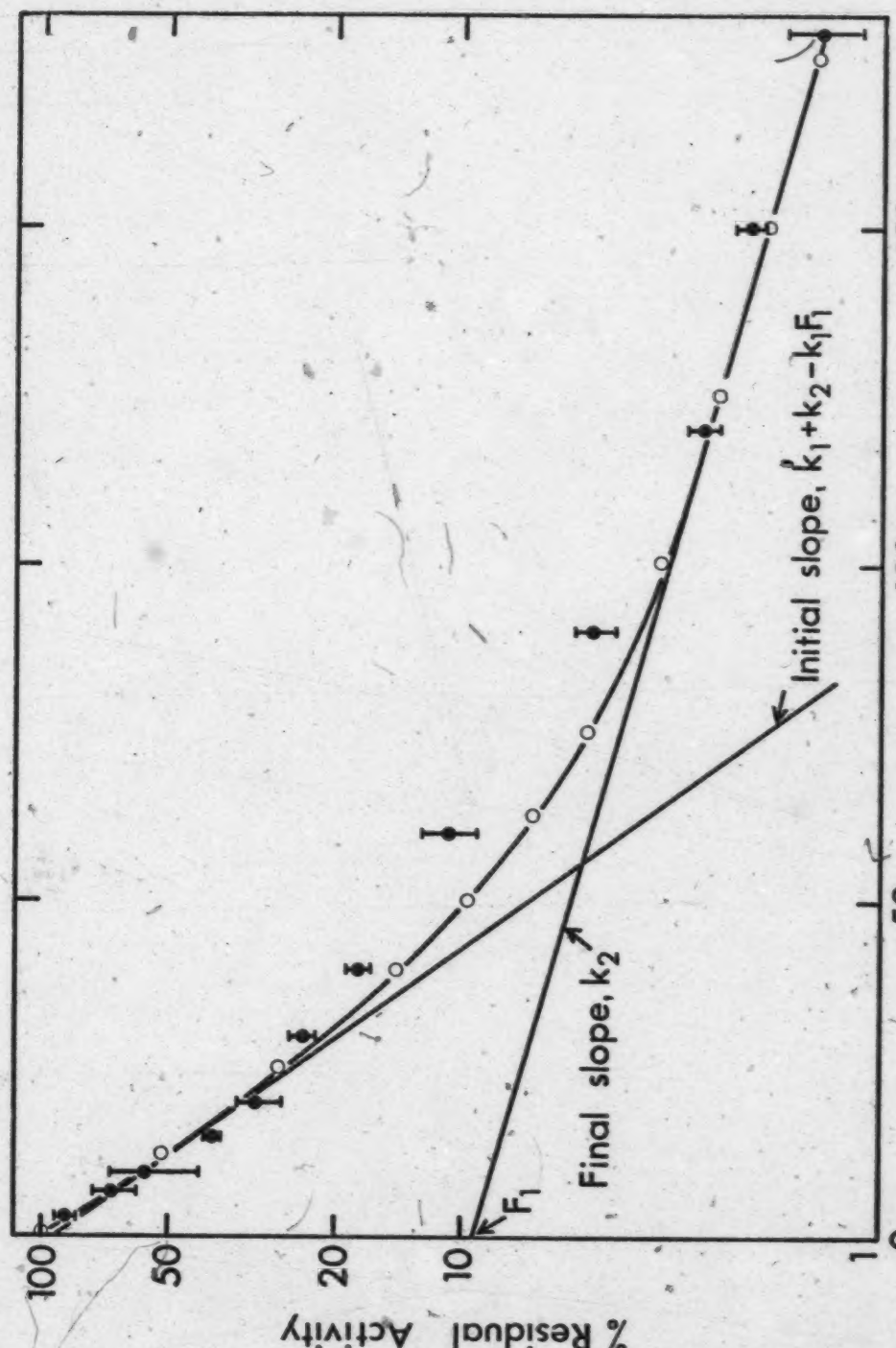

% Residual Activity
100
50
20
10
1
0
50
100
150
Incubation Time (min)
Initial slope, $k_1+k_2-k_1F_1$
Final slope, k_2
F_1

TABLE VII

Evaluation of the pseudo-first-order and second-order rate constants in terms of a model proposing a partially active enzyme species during sulfhydryl modification.

Experiment	(IAA) (mM)	k_1 (min^{-1})	k_2 (min^{-1})	F_1	k_1^1 ($min^{-1}M^{-1}$)	k_2^1 ($min^{-1}M^{-1}$)
1	0.497	0.051	0.012	0.100	102	24.6
2	0.474	0.058	0.011	0.094	123	23.3
3	0.474	0.053	0.008	0.073	111	17.3
Mean of 1 to 3		0.054	0.011	0.089	112	21.9
4*	0.497	0.050	0.010	0.128	100	20.6
Figure 23		0.050	0.011	0.105	y	y

*Determined in the presence of 4.72 mM orotidine.

ySecond-order rate constants were not determined since the means were based on values using two concentrations of IAA that were not identical.

The calculated values of A/Ao, using this equation, agreed well with the mean values determined for the three experiments with a deviation of the calculated value from the mean value being less than 15% over the first 40 minutes of incubation. The deviation of the calculated observed percentage varied by less than 3% after 40 minutes.

This refinement also confirms the fact that both sulfhydryls are freely available to the solvent. The rapid binding of the first sulfhydryl yields a modified protein that has only about 8.9% of the unmodified protein's activity. Orotidine, present at a highly inhibitory level for OMP decarboxylation, did not appreciably alter the inactivation rates of either sulfhydryl group.

Thus it would appear that the two sulfhydryls of OMP decarboxylase that readily react with NEM and IAA are not at the active site of the protein. Their accessibility to the solvent and the resulting reduction of activity upon modification suggests that a conformational change of the protein following reaction with NEM or IAA is the cause of the activity loss. A 0.5 - 1.0 Å displacement of the amino acid residues at the catalytic site due to sulfhydryl reagent binding could completely disrupt the precise space orientation (49) needed at the active site for enzymatic activity.

VIII. CONCLUSION

The enzyme orotidylate decarboxylase (E.C. 4.1.1.23) has not been studied in the past to any great extent. Investigations on it have taken place in passing during pyrimidine analog studies on pyrimidine metabolism (8, 10, 11, 50, 51, 52), and during studies of the rare human disease orotic aciduria (16, 17, 18, 53, 54, 55). In man, the last two enzymes in the synthesis of UMP, OMP pyrophosphorylase and OMP decarboxylase, are thought to be genetically linked (17). In other systems, however, this is not the case, thus showing diversity in the genetics of pyrimidine biosynthesis (20, 56). Studies on the control of pyrimidine biosynthesis in S. cerevisiae (22), in E. coli (19, 20), in Pseudomonas aeruginosa (51), and in Neurospora crassa (58) have revealed an unexpected degree of non-uniformity in the control processes. This makes it highly probable that the individual enzymes in the pathway may have unique properties dependent on the source from which they are isolated.

OMP decarboxylase used in the course of these studies was nearly homogeneous showing only two detectable protein bands on polyacrylamide gels after phosphocellulose column chromatography using 2 mM UMP in the eluting buffer. The protein at this stage displayed remarkable stability to a variety of storage procedures and inactivated very slowly in 80 mM KH_2PO_4, pH 6.10 at room temperature. Magnesium was not essential for the reaction as revealed by the retention of activity upon the addition of EDTA to the assay system. Kinetic assays were carried out at pH 7.0 or 7.5 though the enzyme stability was greater near pH 6. The maximum

velocity and Michaelis constant for OMP were not altered appreciably over a pH range of 6.2 to 7.5, indicating that a hydrogen ion concentration of 3.1×10^{-8} M to 6.3×10^{-7} M does not alter the ionization state of either substrate or protein.

OMP decarboxylase was shown to be a protein probably composed of eight identical subunits, each with a molecular weight of about 19,000. This suggests that each enzyme molecule may have eight catalytic sites. Though the number of active sites actually present was not determined, Hill plots in the presence of neither, either, or both products gave a constant interaction coefficient of unity, indicating an apparent absence of interaction between subunit active sites.

The substrate inhibition observed at higher orotidylate concentrations is therefore likely due to erroneous binding of orotidylate rather than binding to a distinct site on the enzyme molecule. This weak inhibition indicates that this is not a normal method of controlling enzymic activity. Substrate inhibition by OMP and a Hill interaction coefficient of one have been reported previously for this enzyme (6, 7).

The enzyme's specificity is high, with only orotidylic and 6-azaorotidylic acids (10) being known substrates. Orotidine was shown to inhibit the reaction, with 50% inhibition seen at 0.8 mM. However, orotidine was completely inactive as a substrate for decarboxylation emphasizing the requirement of a phosphate at the 5' position of the furanose ring for catalytic activity.

The kinetic study of this enzyme supported an Uni-Bi reaction

mechanism with a dead end complex as shown in Figure 19 of Chapter VI. The maximum velocity in the forward direction was 1.40 umoles/min/ml protein. Each milliliter of protein contained 250 ug in two bands on gel electrophoresis. The essential irreversibility of the reaction makes the calculation of kinetic constants (such as the Michaelis constants of the products) impossible.

Hill plots revealed that the dissociation constant of OMP nearly equaled the value of the Michaelis constant in all cases where:

$$K_d^{OMP} = k_2/k_1 \qquad \text{and}$$

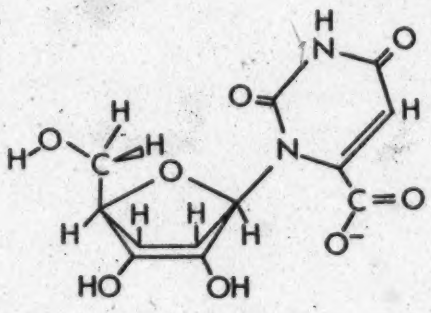
$$K_m^{OMP} = \frac{k_5(k_2 + k_3)}{k_1(k_3 + k_5)}$$

The Michaelis constant approaches the value of the dissociation constant when k_3 is approximately zero. The dissociation constant for a substrate would not be expected to equal the Michaelis constant, especially in the case of an irreversible reaction. This implies that the rate of release of the first product is very slow with respect to the rate of conversion to products without placing limitations on the rate of release of the second product of the enzyme. The apparently slow release of the first product, UMP, makes a deadend complex of UMP with the ternary complex possible. Substrate inhibition seen at concentrations of OMP of 0.22mM or higher might therefore be indicative of an abortive dead end central complex being formed.

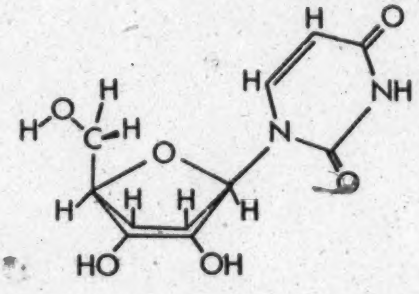
The rapid conversion of orotidylate to uridylate and bicarbonate, and the weakness of the inhibition by the products serve to verify the irreversibility of OMP decarboxylation. This irreversibility ensures the continuous conversion of orotic acid to orotidylic acid where the equilibrium

constant for the OMP pyrophosphorylase reaction is only 0.12 (2). Essential irreversibility of the last step in the biosynthetic pathway of UMP maintains low levels of the intermediary metabolites preventing product inhibition within the pathway. The non-specific inhibition observed with monophosphates may be a weak control in maintaining levels of purine and pyrimidine phosphates in a fixed ratio.

Interpretation of nucleotide inhibition studies may be complex. The question arises as to whether the inhibitors are acting as structural analogs of OMP or of UMP. Orotidine 5'-monophosphate has been reported to exist in the syn conformation due to the bulky nature of the carboxyl group on the C-6 position of the pyrimidine ring (59). Uridine 5'-monophosphate has been shown to exist primarily in the anti-conformation in solution (60). Thus the favored structures for these two nucleotides in aqueous solution are:



OMP



UMP

Nucleotides with the anti-conformation favored in solution probably act as analogs of UMP while nucleotides with a favored syn configuration act as analogs of OMP. The latter should be strict competitive inhibitors of OMP while the former should be non-competitive inhibitors of OMP. Any

ability of such analogs to form a dead end complex with the central complex ($E \cdot OMP \rightleftharpoons E \cdot UMP \cdot HCO_3^-$) would be displayed by curvature of the intercept plots. The ease of transition between syn and anti-configurations for many nucleotides may enable the inhibitor to act as analogs of both OMP and UMP simultaneously, making kinetic interpretation of the inhibition difficult.

The difference in sensitivity to SH reagents shown by OMP decarboxylases from various sources may not be important kinetically. Inactivation of the yeast enzyme is largely a result of conformational changes resulting from the sulfhydryl reagent reacting with two sulfhydryl groups that are both freely accessible to the solvent and in close proximity to the active site. The absence of protection by orotidine at a concentration of 6.4 mM, eight times the concentration that gave 50% inhibition, further substantiates the conclusion that the sulfhydryl groups are not at the active site. This indicates that caution must be used in determining the effects of specific modifying agents on catalytic activity. Destruction of enzymatic activity by such reagents is not conclusive proof of the presence of a given amino acid group at the active or catalytic site of the enzyme. Loss of activity may well be a consequence of configurational changes within the protein at the active or catalytic site due to ligand binding or modification at a site distinct from the active site. An independent means of indicating the involvement of an amino acid group in a catalytic role on the enzyme should be used.

This study has provided some insight into the mode of action of OMP decarboxylase from yeast even though the properties of the enzymic

reaction obviates a complete detailed kinetic study of the reaction in both directions.

BIBLIOGRAPHY

1. Lieberman, I., Kornberg, A., Simms, E. S. J. Am. Chem. Soco. 76: 2844, 1954.

2. Lieberman, I., Kornberg, A., Simms, E. S. J. Biol. Chem. 215: 403, 1955.

3. Blair, D. G. R., Stone, J. E., Potter, Van R. J. Biol. Chem. 235: 2379, 1960.

4. Creasey, W. A., Handschumacher, R. E. J. Biol. Chem. 236: 2058, 1961.

5. Lineweaver, H., Burk, D. J. Am. Chem. Soc. 56: 658, 1934.

6. Appel, S. H. J. Biol. Chem. 243: 3924, 1968.

7. Kasbekar, D. K., Nagabhushanam, A., Greenberg, D. M. J. Biol. Chem. 239: 4245, 1964.

8. Handschumacher, R. E. J. Biol. Chem. 235: 2917, 1960.

9. Smith, S. A., Visser, D. W. J. Biol. Chem. 240: 446, 1965.

10. Cihak, A., Vesely, J., Sorm, F. Coll. Czech. Chem. Comm. 33: 1778, 1968.

11. Smith, D. A., Ray-Burman, P., Visser, D. W. Biochem. Biophys. Acta. 119: 221, 1966.

12. Pynadath, T. L., Fink, R. M. Arch. Biochem. Biophys. 118: 185, 1967.

13. Wolcott, J., Ross, C. Biochem. Biophys. Acta. 122: 532, 1966.

14. Nakayama, K., Tanaka, H. Agr. Biol. Chem. 35: 518, 1971.

15. Rochovansky, O. Arch. Biochem. Biophys. 138: 574, 1970.

16. Smith, L. H., Sullivan, M., Huguley, C. M. J. Clin Invest. 40: 656, 1961.

17. Smith, L. H., Huguley, C. M., Bain, J. A. In: "The Metabolic Basis of Inherited Disease," J. B. Stanburg, J. W. Wyngaarden and D. S. Frederison, eds., p. 739, McGraw-Hill, Inc., New York, 1966.

18. Pinsky, L., Krooth, R. S. Proc. Natl. Acad. Sci. 57: 1267, 1967.

19. Smith, L. H., Lotz, M. J. Lab. & Clin. Med. 61: 211, 1963.

20. Beckwith, J. R., Pardee, A. B., Austrian, R., Jacob, F. J. Mol. Biol. 5: 618, 1962.

21. Taylor, A. L., Beckwith, J. R., Pardee, A. B., Austrian, R., Jacob, F. J. Mol. Biol. 8: 771, 1964.

22. Lacroute, F. J. Bact. 95: 824, 1968.

23. Grenson, M. Eur. J. Biochem. 11: 249, 1969.

24. Lue, P., Kaplan, J. G. Can. J. Biochem. 49: 403, 1971.

25. Fox, R. M., Wood, M. H., O'Sullivan, W. J. J. Clin. Invest. 50: 1050, 1971.

26. Yoshikawa, M., Kato, T., Takenishi, T. Tetrahedron Lett. 50: 5065, 1967.

27. Yoshikawa, M., Kato, T. Bull. Chem. Soc. Japan. 40: 2849, 1967.

28. Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Masuda, T., Honjo, M. J. Org. Chem. 34: 1547, 1969.

29. Fox, J. J., Yung, N., Wempen, I. Biochem. Biophys. Acta. 23: 295, 1957.

30. Layne, E. In: "Methods of Enzymology," N. O. Kaplan and S. P. Colowick, eds., III, p. 447, Academic Press, New York, 1957.

31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. J. Biol. Chem. 193: 265, 1951.

32. Koshland, D. E., Levitzky, A. Biochem. Biophys. Acta. 206: 473, 1970.

33. Ornstein, L. Ann. N.Y. Acad. Sci. 121: 321, 1964.

34. Weber, K., Osborn, M. J. Biol. Chem. 244: 4406, 1969.

35. Peller, L., Alberty, R. A. J. Am. Chem. Soc. 81: 5907, 1959.

36. Cleland, W. W. Biochem. Biophys. Acta. 67: 104, 1963.

37. Cleland, W. W. Biochem. Biophys. Acta. 67: 173, 1963.

38. Cleland, W. W. Biochem Biophys. Acta. 67: 188, 1963.

39. Blair, D. G. R., Potter, Van R. J. Biol. Chem. 236: 2503, 1961.

40. Wilkinson, G. N. Biochem. J. 80: 324, 1961.

41. Hill, A. V. J. Physiol. (London). 40: 190, 1910.

42. Circular OR-10, Ultraviolet Absorption Spectra of 5'-Ribonucleotides, 1956, Pabst Laboratories, Division of Pabst Brewing Company, Milwaukee, Wisconsin.

43. Fox, R. M., Royse-Smith, D., O'Sullivan, W. J. Science 168: 861, 1970.

44. King, E. L., Altman, C. J. Phys. Chem. 60: 1375, 1956.

45. Roseveare, W. E. J. Am. Chem. Soc. 53: 1651, 1931.

46. Battell, M. L., Zarkadas, C. G., Smillie, L. B., Madsen, N. B. J. Biol. Chem. 243: 6202, 1968.

47. Hasinoff, B. B., Madsen, N. B., Auramovic-Zikic, O. Can. J. Biochem. 49, 742, 1971.

48. Ray, W. J., Koshland, D. E. J. Biol. Chem. 233: 1973, 1961.

49. Storm, D. R., Koshland, D. E. Proc. Natl. Acad. Aci. 66: 445, 1970.

50. Brueman, N., Holland, J., Sheehe, P. Can. Res. 22: 113, 1962.

51. Pasternak, C., Handschumacher, R. E. J. Biol. Chem. 234: 2992, 1959.

52. Conn, H., Creasey, W., Calabresi, P. Can. Res. 27: 618, 1967.

53. Fallon, H., Frei III, E., Block, J., Seegmillar, J. J. Clin. Invest. 40: 1906, 1961.

54. Fallon, H. J., Lotz, M., Smith, L. H. Blood. 20: 700, 1962.

55. Fox, R. M., O'Sullivan, W. J., Firkin, B. Am. J. Med. 47: 332, 1969.

56. Caroline, D. F. J. Bact. 100: 1371, 1969.

57. Isaac, J., Holloway, B. J. Bact. 96: 1732, 1968.

58. Caroline, D. F., Davis, R. J. Bact. 100: 1378, 1969.

59. Hruska, F. E. J. Am. Chem. Soc. 93: 1975, 1971.

60. Donohue, J., Trueblood, K. N. J. Mol. Biol. 2: 363, 1960.